Towards an Understanding of the Evolution of the HPV E6 PDZ Binding Motif

Thesis

TOWARDS AN UNDERSTANDING OF THE EVOLUTION OF THE HPV E6 PDZ BINDING MOTIF

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ABSTRACT

The high-risk human alpha papillomaviruses, associated with the development of cervical cancer differ in the carboxyl-terminal sequence of the E6 oncogenic protein, where a PDZ-binding motif (PBM) is located. The PBM binds to PDZ-containing proteins of the cell, and variation in the PBM sequence is likely to affect the PDZ protein selectivity of E6, and the phospho-acceptor site embedded within the PBM. Using the E6 proteins from HPV types with diverse phylogenetic and risk classifications, we found that differences in their PBMs are reflected in their substrate selection. Moreover, we show that the ability of E6 proteins to target PDZ proteins has co-evolved with the phospho-regulation of the PBMs.

Previously identified residues known to reduce both HPV-18 phosphorylation and its functional flexibility in substrate selection, were mutated in the core and upstream regions of HPV-66 and HPV-40 E6 PBMs to generate phospho-acceptor sites for CHK1, PKA and AKT kinases. The patterns of binding to PDZ proteins by these mutants were then evaluated. I found that the last residue of HPV-18 E6 (Valine) is critical for its recognition of MAGI-1 and when the last residue of HPV-40 E6 (Cysteine) is mutated to Valine, it increases HPV-40 E6's PBM-dependent binding to MAGI-1 in pull-down assays. This was also confirmed with Dlg1. As expected, the reverse (Valine to Cysteine) substitution in the HPV-18 E6 PBM also destroys its binding to Scribble, but the Cysteine to Valine substitution failed to confer Scribble binding on HPV-40, suggesting that additional critical residues are required for Scribble recognition. Additionally, we evaluated the phosphorylation of the HPV-66 E6 protein by the three kinases, introducing arginine residues on the p-4 and p-5 positions of the PBM. The wild-type HPV-66 E6 protein is not phosphorylated, as well as the p-5 mutant. On the contrary, mutations on p-4 and p-4/p-5 positions have increased phosphorylation by PKA, slightly by CHK1 but not by AKT. Mutations on p-5 position resulted in a mild increase in recognition of MAGI-1 whilst surprisingly mutations on p-4 which confers phosphorylation, does not. The arginine residue substitutions resulted in a mild increase of the ability of the HPV-66 E6 PBM to bind Dlg1, and failed to confer interaction with Scribble; again, suggesting the involvement of additional residues.

Moreover, we used HPV-16 E6 protein to further evaluate if residues upstream of the PBM are important for binding PDZ proteins and whether upstream phosphor acceptor sites might regulate PDZ recognition. We evaluated a threonine residue on p-6 position and found that this position is phosphorylated by CK1 kinase with lower

efficiency. Furthermore, the Aspartate substitution mildly increased binding to TIP-1 and SNTB2 PDZ proteins, and conversely, the Alanine substitution significantly decreased binding. Furthermore, we observed that a triplet of serine residues on positions p-8, p-9 and p-13 upstream of the PBM can be multiply phosphorylated by PKC and CK1 kinases. The multiple Aspartate substitution mutant showed higher retention of E6 in the cytoplasm, and the Alanine substitution mutant higher retention in the nucleus, indicating that phosphorylation of these residues might modulate E6 localisation. Taken together these studies demonstrate a unique linkage between evolution of the phospho-acceptor site embedded in the PBM and functional flexibility, together with further regulation provided by potentially unique phospho-acceptor sites only found in HPV-16 E6.

Finally, we evaluated the capacity of the low-risk HPV-11, a less potent version of the HR types, but a common cause of benign tumours in the anogenital region and the upper respiratory tract, rarely associated with cancer, to degrade p53. Although, 11E6 can bind E6AP and p53, it binds the carboxy-terminal site of p53, and not the core domain that is necessary for degradation induced by the E6 of high-risk types. Regardless, a recent report has shown that HPV-11 E6 degrades p53 under high-confluency conditions, suggesting a conserved and common mechanism between high- and low-risk types. We showed in Part 3, that HPV-11 E6 degrades p53 after treatment with etoposide, a DNA damaging agent. It has been shown that Etoposide induces the phosphorylation of p53 on serine 15, an important and nucleating event in p53 activation through post-translational modifications. Furthermore, 11E6 induces the degradation of the p53 phospho-mimic mutant S15D, which mimics the activated form of p53 and which is not susceptible to Mdm2-induced degradation. Moreover, we confirmed that 11E6 co-localizes with p53 after the treatment with Etoposide, and with the p53 S15D phospho-mimic mutant. Interestingly, we observed that 11E6 influences the cellular distribution of wild type p53 to the cytoplasm, when transiently co-transfected together, as with the phospho-mimic mutant. We hypothesized that the common ancestor of high- and low-risk types might have been able to degrade specific forms of p53, and that eventually, the high-risk types developed the ability to degrade all forms of p53, contributing to their oncogenic potential. Whether 11E6's specific ability to induce degradation of Ser15 phospho-p53 is related to HPV-11's successful replication and maintenance within the cell needs investigating.

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LIST OF ACRONYMS

AF-6 - Afadin, Adherens Junction Formation Factor-6

AKT – Serine/threonine Kinase 1 (protein kinase B)

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATM - Ataxia-Telangiectasia Mutated

ATR - Ataxia Telangiectasia and Rad3-Related Protein

BAK - BCL2 Antagonist/Killer

CamKII – Calcium/calmodulin dependent Protein Kinase II

CBP – cAMP response elements (CREB)-binding protein

CDK – Cyclin Dependent Kinase

CHK1 – Checkpoint Kinase 1

CHK2 - Checkpoint Kinase 2

CIN – Cervical Intraepithelial Neoplasia

CK1 – Casein Kinase 1

CK2 – Casein Kinase 2

DAPI - 4′,6-diamidino-2-phenylindole

DDR – DNA Damage Response

DLG - Discs Large MAGUK Scaffold Protein

DNA-PK – DNA-dependent Protein Kinase

DSB – Double Strand Break

E6AP – E6-associated Protein

EDD - Ubiquitin Protein Ligase E3 Component N-Recognin 5 (UBR5)

EGF – Epidermal Growth Factor

ERBIN - Erbb2 Interacting Protein

ERK – Extracellular Signal-regulated Kinase

ETO - Etoposide

EV - Epidermodysplasia Verruciformis

GSK3 – Glycogen Synthase Kinase 3

GST – Glutathione S-Transferase

HA – Hemagglutinin (antibody)

HaCaT - Cultured Human Keratinocyte cells

HEK293 - Human Embryonic Kidney 293 cells

HERC2 - HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2

HPV - Human papillomavirus

HR - High Risk

HTERT – Human Telomerase Reverse Transcriptase

Kir2.3 - Potassium Inwardly Rectifying Channel Subfamily J Member 4

LR - Low Risk

MAGI-1 - Membrane Associated Guanylate Kinase, WW And PDZ Domain Containing 1

MAML - Mastermind Like Transcriptional Coactivator 1

MAPK – Mitogen-Activated Protein Kinase

MCM – Minichromosome Maintenance Protein Complex

MDM2 – Mouse Double Minute 2 homolog

MUPP1 - Multiple PDZ Domain Crumbs Cell Polarity Complex Component

NES – Nuclear Export Signal

NHERF - Na+/H+ Exchange Regulatory Co-Factor

NLS – Nuclear Localisation Signal

NOC - Nocodazole

ORF – Open Reading Frame

PAK - P21 (RAC1) Activated Kinase

PATJ – Protein Associated with Tight Junctions

PBM – PDZ – Binding Motif

PDZ – PSD-95-Dlg-ZO-1

PI3K - Phosphatidylinositol-4,5-Bisphosphate 3-Kinase

PKA – cAMP-dependent protein kinase

PKC –Protein Kinase C

PKN –Protein Kinase N

pRB – Retinoblastoma Protein

PSD-95 - Post-Synaptic Density Protein 95

PTEN - Phosphatase and Tensin Homolog

PTM – Post Translational Modifications

RACK1 - Receptor for Activated C Kinase 1

RPP - Recurrent Respiratory Papillomatosis

RSK1 - Ribosomal Protein S6 Kinase A1

SCRIB - Scribble Planar Cell Polarity Protein

SNTB2 - Syntrophin Beta 2

SNX27 – Sortin Nexin 27

SSB – Single Strand Break

TENI - Teniposide

TET – Tetramerization Domain

TIP-1 - Tax1 (Human T-Cell Leukemia Virus Type I) Binding Protein 3

UBR4 - Ubiquitin Protein Ligase E3 Component N-Recognin 4

UT - Untreated

VLPs – Virus Like Particles

WNK4 - WNK Lysine Deficient Protein Kinase

ZO – Zona Occludens

CHAPTER 1: INTRODUCTION

Persistent infection with high-risk human alpha papillomaviruses is associated with the development of cervical cancer (Durst et al., 1983; Zur Hausen, 1976; Zur Hausen et al., 1981), causing more than 500 000 new cases of invasive cervical cancer annually worldwide (approximately 569 847 in 2018), and resulting in approximately 300 000 death annually (311 365 deaths in 2018) (Bray et al., 2018; Bruni et al., 2019; Formana et al., 2012; Muñoz et al., 2006; Serrano et al., 2018). Indeed, cervical cancer is the third leading cause of female cancer in the world, and the second most common female cancer in women between 15 to 44 years of age in the world. The rates of cervical cancer vary greatly between different geographical regions, mainly due to the combined effects of differing prevalence of cervical HPV infection and variability in the quality of the local cervical cancer screening programs. HPV epidemiology is complex, since the data reported relies primarily on HPV DNA testing, therefore the data on HPV prevalence depends on the sensitivity of the HPV assay used and the frequency of testing. Additionally, HPV prevalence is a function of the incidence and the evident persistence of infection. Prevalence has also been shown to be highly variable by geographical region, age, and dependent on different factors, particularly sexual behavior (Bruni et al., 2019).

The WHO has defined at least 12 different HPV types as being cancer-causing in the cervix (Bruni et al., 2019). HPV-16 is the most frequent high-risk type detected and is the predominant type causing invasive cervical cancer worldwide (~ 60%), followed by HPV-18 (~15%). Other cancer-causing genotypes, such as HPV-31, HPV-33 and HPV-58 are generally less predominant than HPV-16 and -18, but in some countries these genotypes are even more prevalent. In addition, HPV-16 is responsible for the majority of all HPV-related, non-cervical cancers (85%) (Bruni et al., 2010; Ciapponi et al., 2011)

Although all high-risk HPV types have similar genome organization, there are differences in their protein functions, their ability to infect different types of epithelial tissue, and protein expression patterns that affect the phenotype of the disease (Doorbar et al., 2012; Meisal et al., 2017; Schiffman et al., 2016). The nature of the initially infected cell and how that relates to disease outcome is still a matter of speculation for most carcinogenic viruses of the high-risk group 1 (HPV types -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, and -59), the probably carcinogenic group 2A (HPV-68) and the possibly carcinogenic group 2B (HPV types -26, -53, -66, -67, -70, -73, and -82) (Bruni et al., 2019; Meisal et al., 2017). The precise role of the E6

oncoprotein of these various HPV types in infected basal cells and in keratinocytes; and its contribution to the ability of the viruses to infect different kinds of tissues is not yet defined, and could affect the persistence of the infection (Chan et al., 2013).
Prophylactic vaccination is the most long-term effective intervention for the control of high-risk HPV infections and HPV-related cancers (de Sanjose et al., 2019). The available HPV vaccines consist of L1-containing VLPs, which have the form of the native virus particle but lack DNA, thus being non-infectious. Currently, there are three highly efficient VLP-based HPV vaccines: the bivalent Cervarix (GlaxoSmithKline (GSK), London, UK) which contains HPV-16 and HPV-18; the quadrivalent Gardasil, which includes HPV-6 and -11 in addition to types -18 and -16; and the nonavalent Gardasil9 (both Merck, Kenilworth, New Jersey, USA), which provides protection against HPV types -6, -11, -16, -18, -31, -33, -45, -52 and -58 (Bruni et al., 2019; Huh et al., 2017).

Low-risk Human papillomavirus

The low-risk HPV types are the majority of the more than 200 known human papillomaviruses (Alpha, Beta, Gamma, Mu and Nu). They cause benign hyper proliferative lesions, including genital warts (HPV-6 and -11), common warts (HPV-2, -27, -57), flats warts (HPV-3 and -10), verrucas or myrmecia (HPV-1) as well as many other skin lesions and are not a frequent cause of malignant carcinoma among the general population, since in most cases lesions will eventually be cleared by a cell mediated immune response (Egawa & Doorbar, 2017; Sterling et al., 2001).

Alpha-low-risk types, particularly HPV-6 and HPV-11 are etiologically associated with genital warts, such as condylomata acuminate (CA) (Bruni et al., 2019; Jamshidi et al., 2012), which have become one of the most widespread sexually transmitted diseases, with an annual global incidence of 160 to 289 cases per 100 000 . Particularly, HPV-11 is the most common cause of the two most frequent benign tumours in the anogenital region and upper respiratory tract: associating approximately with 20% of anogenital warts and 30 to 40% of laryngeal papillomas, respectively (Bruni et al., 2019; F. et al., 2008; Gillison et al., 2012; Jelen et al., 2016; Komloš et al., 2012). Furthermore, it is present in 0.5% of samples from HPV-positive women with a normal cytology worldwide and causes 2.3% of cervical low-grade squamous cell intraepithelial lesions (Bzhalava et al., 2013; Prétet et al., 2008). It is

generally considered a low-risk type due to its rare presence in HPV-related cancer in humans, especially cervical cancer. However, it has become a serious threat to public health, with high recurrences and the need of multiple surgical procedures in infected patients .

Despite their low-risk designation, low-risk types can cause problematic pathologies in susceptible populations, including recurrent respiratory papillomatosis (RRP), and in these cases they can be associated with development of cancer (Egawa & Doorbar, 2017). HPV-6 and -11 as well as other low-risk types including HPV-42, -44 and -70 DNA are sometimes detected in invasive cervical and anal cancer, but it is quite rare, thus not been considered a significant cause for malignant transformation in the general population (Cornall et al., 2013; Guimerà et al., 2013; Li et al., 2011). Moreover, the link between low-risk types and carcinoma remains unclear except in immunosuppressed individuals and in certain genetic backgrounds, in which the progression route towards cancer is different.

Particularly, there are rare cases of HPV-11-positive cases of cervical and anal squamous cell carcinomas, malignantly transformed laryngeal papillomas and sinonasal inverted papillomas associated with squamous cell carcinoma .

Moreover, HPV11 has also been associated with recurrent respiratory papillomatosis (RRP) . In susceptible individuals, laryngeal papillomas can persist for decades, re-growing after surgical removal, and in some instances, giving rise to metastatic lesions in the lower airway and lung . Due to its clinical relevance and its possible association with cancer, it has been included in the vaccine platform currently established (Bruni et al., 2019).

Papillomavirus evolution

Papillomaviruses have co-evolved with their animal host species, including humans, over millions of years from common ancestors into specific virus-host ecosystems. The phylogenetic trees that have been reported are linked to host and tissue tropisms, biological behavior and carcinogenic potential. More than 200 human papillomavirus genotypes are known, and these are categorized into phylogenetic genera and numbered species (Bruni et al., 2019; De Villiers et al., 2004). Each HPV type is

evolutionarily adapted to a particular human epithelial tissue, such as anogenital skin and mucosa (Bruni et al., 2019; Kovacic et al., 2006).

The major evolutionary branches of HPVs are the Alpha, Beta and Gamma genera. These viruses have evolved to infect specific epithelia with specific survival strategies (Chen et al., 2005; Doorbar et al., 2012; Schiffman et al., 2016) (Figure 1). Many HPV types in the Beta and Gamma genera are associated with inapparent infections of the skin that are acquired in early childhood and can persist, producing low levels of virus particles, over years and decades. By contrast, Mu HPVs infect palmar and plantar epithelial sites and produce highly productive deep warts that are cleared by a cellular immune response often after months or even years (Schiffman et al., 2009).

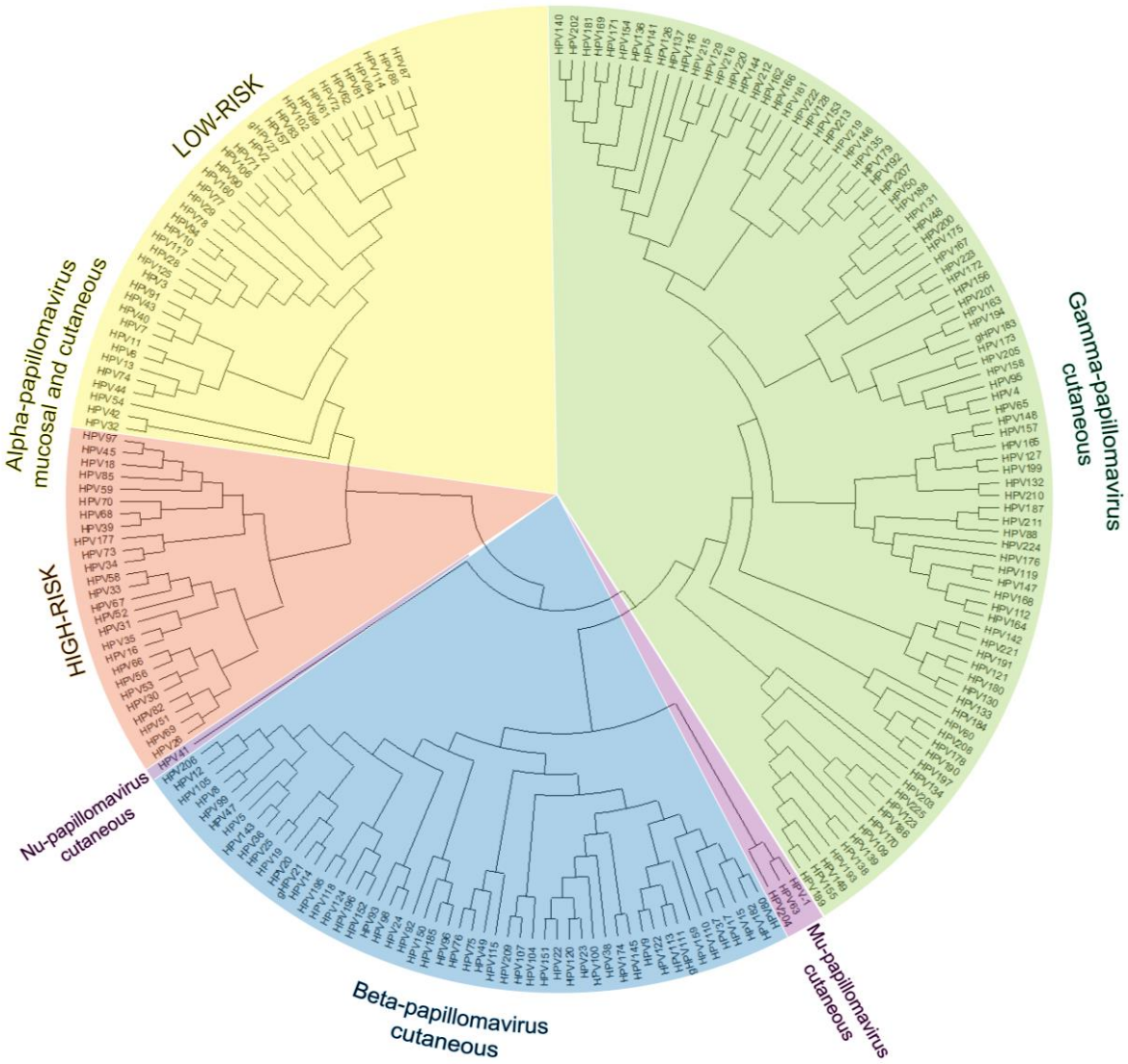


Figure 1.- The human papillomaviruses phylogenetic classification tree. The HPV types fall into five genera, with the Alpha and the Beta/Gamma genera representing the largest groups. Human papillomaviruses types from the Alpha genus are often classified as low-risk (yellow) or high-risk (orange) and can be cutaneous and mucosal. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura et al., 2004). This analysis involved 214 nucleotide sequences obtained from PaVE website (http://pave.niaid.nih.gov). The evolutionary analyses were conducted in MEGA X , using the complete E6 gene sequences.

The Alpha genus is mostly composed of HPV types with mucosal tropism (α1, α3, α5, α6, α7, α8, α9, α10, α11, α13, α14), which can be sexually transmitted, and HPV types with cutaneous tropism (α2, α4, α8) (Egawa et al., 2015). In most cases they are controlled by the immune system, but when they persist they can induce cervical lesions and cancer; as well as rarer cancers of non-keratinized mucosa and skin of the lower genital tract, including the vagina, vulva and penis; and the anus and the oropharynx (Bruni et al., 2019). The topology of the alpha-HPV phylogenetic tree strongly predicts viral behavior (Burk et al., 2009; Schiffman et al., 2005). The phylogenetic tree based on L1 protein alignments includes three branches/clades; two of which contain the low-risk types that can persist without any apparent pathology, or that cause highly productive warts; the third important evolutionary high-risk branch/clade contains the HPV types with oncogenic potential. Moreover, when the E6 sequences are used for generating the alignment, a clear two-clade separation in low-risk and high-risk types is observed (Figure 2). The single high-risk clade is composed of five species groups: α-5, α-6, α-7, α-9, and α-11, with different risk profiles (Bernard et al., 2010; De Villiers et al., 2004). The α-9 species is the most important, consisting almost entirely of carcinogenic types (e.g. HPV-16), but having individual types that show very large differences in risk of viral persistence, precancer and eventually cancer (e.g. HPV-67). This has led to a further level of classification based on the risk of causing cancer as: carcinogenic (Group 1), probably/possibly carcinogenic (Group 2) and low-risk non-carcinogenic (Group 3) (Bruni et al., 2019). Additionally, the previous risk classification is still used: high-risk/carcinogenic (Group 1), probably carcinogenic (Group 2A), possibly carcinogenic (Group 2B) and low-

risk/not associated with cancer (Group 3) (Bruni et al., 2019; Meisal et al., 2017; Schiffman et al., 2009) (Figure 2; Table 1).

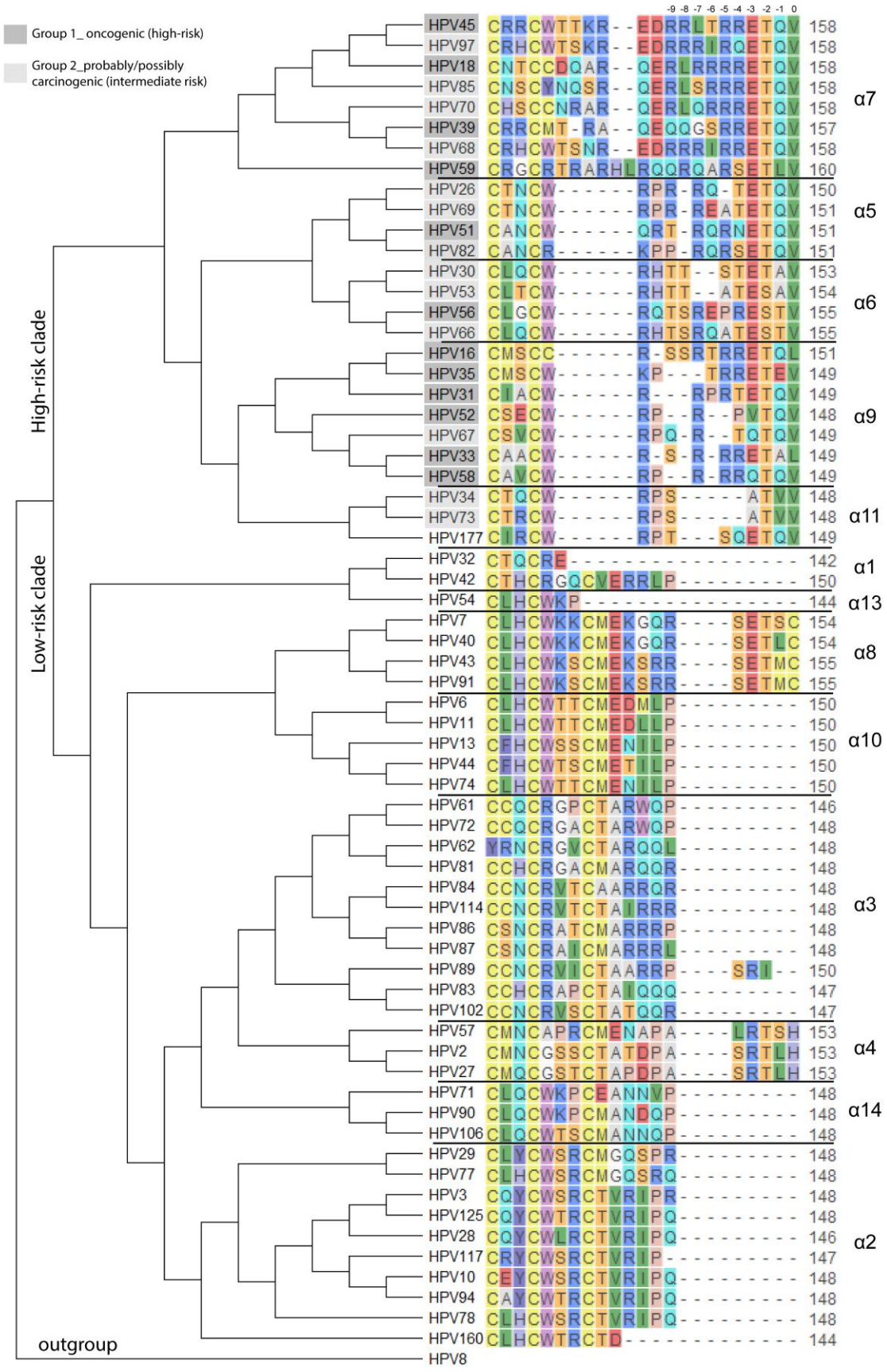

Group 1_ oncogenic (high-risk)
Group 2_probably/possibly carcinogenic (intermediate risk)
High-risk clade
Low-risk clade
outgroup
-9 -8 -7 -6 -5 -4 -3 -2 -1 0
HPV45 CRRCWTTKR--EDRRLTRRETQV 158
HPV97 CRHCWTSKR--EDRRRIRQETQV 158
HPV18 CNTCCDQAR--QERLRRRRETQV 158
HPV85 CNSCYNQSR--QERLSRRRETQV 158
HPV70 CHSCCNRAR--QERLQRRRETQV 158
HPV39 CRRCMT-RA--QEQQGSRRETQV 157
HPV68 CRHCWTSNR--EDRRRIRRETQV 158
HPV59 CRGCRTRARHLRQQRQARSETLV 160
α7
HPV26 CTNCW------RPR-RQ-TETQV 150
HPV69 CTNCW------RPR-REATETQV 151
HPV51 CANCW------QRT-RQRNETQV 151
HPV82 CANCR------KPP-RQRSETQV 151
α5
HPV30 CLQCW------RHTT--STETAV 153
HPV53 CLTCW------RHTT--ATESAV 154
HPV56 CLGCW------RQTSREPRESTV 155
HPV66 CLQCW------RHTSRQATESTV 155
α6
HPV16 CMSCC------R-SSRTRRETQL 151
HPV35 CMSCW------KP---TRRETEV 149
HPV31 CIACW------R---RPRTETQV 149
HPV52 CSECW------RP--R--PVTQV 148
HPV67 CSVCW------RPQ-R--TQTQV 149
HPV33 CAACW------R-S-R-RRETAL 149
HPV58 CAVCW------RP--R-RRQTQV 149
α9
HPV34 CTQCW------RPS-----ATVV 148
HPV73 CTRCW------RPS-----ATVV 148
HPV177 CIRCW------RPT---SQETQV 149
α11
HPV32 CTQCRE---------------- 142
HPV42 CTHCRGQCVERRLP-------- 150
α1
HPV54 CLHCWKP--------------- 144
α13
HPV7 CLHCWKKCMEKGQR----SETSC 154
HPV40 CLHCWKKCMEKGQR----SETLC 154
HPV43 CLHCWKSCMEKSRR----SETMC 155
HPV91 CLHCWKSCMEKSRR----SETMC 155
α8
HPV6 CLHCWTTCMEDMLP-------- 150
HPV11 CLHCWTTCMEDLLP-------- 150
HPV13 CFHCWSSCMENILP-------- 150
HPV44 CFHCWTSCMETILP-------- 150
HPV74 CLHCWTTCMENILP-------- 150
α10
HPV61 CCQCRGPCTARWQP-------- 146
HPV72 CCQCRGACTARWQP-------- 148
HPV62 YRNCRGVCTARQQL-------- 148
HPV81 CCHCRGACMARQQR-------- 148
HPV84 CCNCRVTCAARRQR-------- 148
HPV114 CCNCRVTCTAIRRR-------- 148
HPV86 CSNCRATCMARRRP-------- 148
HPV87 CSNCRAICMARRRL-------- 148
HPV89 CCNCRVICTAARRP----SRI-- 150
HPV83 CCHCRAPCTAIQQQ-------- 147
HPV102 CCNCRVSCTATQQR-------- 147
α3
HPV57 CMNCAPRCMENAPA----LRTSH 153
HPV2 CMNCGSSCTATDPA----SRTLH 153
HPV27 CMQCGSTCTAPDPA----SRTLH 153
α4
HPV71 CLQCWKPCEANNVP-------- 148
HPV90 CLQCWKPCMANDQP-------- 148
HPV106 CLQCWTSCMANNQP-------- 148
α14
HPV29 CLYCWSRCMGQSPR-------- 148
HPV77 CLHCWSRCMGQSRQ-------- 148
HPV3 CQYCWSRCTVRIPR-------- 148
HPV125 CQYCWTRCTVRIPQ-------- 148
HPV28 CQYCWLRCTVRIPQ-------- 146
HPV117 CRYCWSRCTVRIP--------- 147
HPV10 CEYCWSRCTVRIPQ-------- 148
HPV94 CAYCWTRCTVRIPQ-------- 148
HPV78 CLHCWSRCTVRIPQ-------- 148
α2
HPV160 CLHCWTRCTD---------- 144
HPV8

Figure 2.- The topology of the alpha-HPV phylogenetic tree strongly predicts viral behavior. The alpha types are divided in two clades based on E6 sequence alignments. The species are annotated on the right, and the carboxy-terminal amino-acid sequence of E6 is shown in colors. Within the high-risk clade, the types from carcinogenic group 1 are highlighted in black, and the probably/possibly carcinogenic types from group 2, in gray. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Maximum Composite Likelihood method. This analysis involved 65 nucleotide sequences of the alfa-HPV types and HPV-8 as the outgroup. Evolutionary analyses were conducted in MEGA X (Felsenstein, 1985; Kumar et al., 2018; Nei & Saitou, 1987; Tamura et al., 2004). Although the alignment and the topology tree were generated, the figure was adapted from Van Doorslaer et al 2015.

ICO/IARC HPV Information Centre 2019				
	Group	Description	Types	Phylogenetic classification
Oncogenic HPV types	Group 1	High-risk HPV types	16, 31, 33, 35, 52, 58 18, 39, 45, 59 56 51	α9 α7 α6 α5
	Group 2	Probably/Possibly carcinogenic	67 70, 68, 97, 85 66, 30, 53 26, 69, 82 34, 73	α9 α7 α6 α5 α11
Non-oncogenic HPV types	Group 3	Low-risk types	42, 32 89, 83, 84, 87, 86, 81, 61, 62, 72 57 40, 43, 91 6, 11, 44, 55, 74 54 90, 71 64	α1 α3 α4 α8 α10 α13 α14 -
Bruni L, Albero G, Serrano B, Mena M, Gómez D, Muñoz J, Bosch FX, de Sanjosé S. ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the World. Summary Report 17 June 2019.				

IARC 2012			
Group	Description	Types	Phylogenetic classification
Group 1	Carcinogenic (High-risk HPV types)	16, 31, 33, 35, 52, 58 18, 39, 45, 59 56 51	α9 α7 α6 α5
Group 2A	Probably carcinogenic (High-risk HPV types)	68	α7
Group 2B	Possibly carcinogenic (Intermediate risk)	67 70 66, 53 26, 82 73	α9 α7 α6 α5 α11
Group 2B	Unknown risk	97 30 69 86 34, 85	α7 α6 α5 α3 α11
Group 3	Low-risk types	42 81, 89, 61, 72 40, 43 6, 11, 44 90, 71	α1 α3 α4 α8 α13
	Low-risk types Unknown risk (no IARC 2012)	83, 87 91 74	α3 α4 α8
Meisal et al., 2017			

Table 1.- The alpha-HPV risk classification. The alfa-types species are most recently divided in groups 1, 2, and 3 (Top). The previously reported IARC risk classification subdivides the group 2 in groups 2A- probably carcinogenic, group 2B- possibly carcinogenic, and group 2B-unknown risk.

Originally, these high-risk types within the groups of species were distinguished by more than 10% diversity in the sequence of the conserved L1 gene; but after the expansion of the population, the geographical dispersion and the time scale have contributed to the evolution of HPV types mainly through single-nucleotide variation into variants or variant lineages (Schiffman et al., 2010; Schiffman & Wentzensen, 2010). Next generation sequencing has made it possible to fine-tune the phylogenetic arrangement and epidemiology of HPV, demonstrating that each HPV type is a grouping of closely related viruses that can be further organized into defined evolutionary branches called variants, sub-variants and isolates, that differ by less than 3% in the nucleotide sequence and which can have different carcinogenic potential (Bernard et al., 2006; Burk et al., 2013; Chen et al., 2011; De Villiers et al., 2004; Sichero & Villa, 2006).

In general, HPV-16 and HPV-18 variants confer different risks of viral persistence and progression to precancer or cancer. The Asian-American (AA) or African (Af) HPV-16 variants have a three-fold greater risk of cervical cancer compared with European (E) variants; and non-European variants of HPV-18 may be more common in cancer tissues and high-grade cervical lesions (Chen et al., 2005; Hildesheim et al., 2001; Schiffman et al., 2010). In the specific case of the Alpha-9 group, the variants differ in risk of persistence. For some HPV genotypes, variant lineages or sub-lineages like HPV-35 A1 differ in their risk of CIN3+ (Koshiol et al., 2009). Nevertheless, there is almost no data on the natural history of other high-risk HPV variants, and it is not yet known if variants from other species and types might confer different risks of viral persistence or lesion progression. HPV types with moderate, limited, carcinogenicity could have one or more variants that are definitively carcinogenic (Burk et al., 2013; Chen et al., 2014; Chen et al., 2011; Schiffman et al., 2010).

Furthermore, HPV-11 has also acquired nucleotide changes that have fixated, generating lineages which are still evolving. Currently, the phylogenetic analysis confirmed the existence of two HPV11 variant groups (Burk et al., 2011; Jelen et al.,

2016; Maver et al., 2011). These were classified into two sub lineages: A1 (prototypic variant) and A2. Being the isolates from sub lineage A2 the most prevalent globally .

Genomic organization of papillomaviruses

All papillomaviruses have a common genome organization and rely on infection of a differentiating epithelium to complete their productive life cycle. The virus particles have a non-enveloped icosahedral capsid made up of 360 copies of L1 protein, containing a double-stranded circular DNA genome of approximately 8 000 base pairs (Bruni et al., 2019; Schwarz et al., 1985). The L1 capsid and the DNA content are linked by a smaller number of L2 proteins (Buck et al., 2005). Papillomavirus genomes contain only eight to nine open reading frames. After infection, viral gene expression is controlled by multiple promoters that allow different viral gene products to be expressed at different stages during the viral life cycle (Buck et al., 2008).

Papillomavirus gene products can be arranged into core and accessory proteins. The core proteins, E1, E2, L1 and L2, are highly conserved among papillomaviruses (Blakaj et al., 2009). These participate in viral genome replication (E1 and E2) and virus assembly (L1 and L2), where the ‘E’ indicates ‘early’ and the ‘L’ indicates ‘late’, based on when transcription occurs during the viral life cycle (Figure 3, Table 2). In contrast, the accessory proteins E4, E5, E6 and E7 show greater variability in both the time of expression and their functional characteristics. These proteins modify the infected cell to facilitate virus replication and, depending on the HPV type, they have different disease association (Schiffman et al., 2016).

The E6 and E7 oncoproteins are essential in the viral life cycle, increasing virion production by driving cell cycle re-entry and genome amplification in the differentiating epithelial layers. Even though this process is the same in both high-risk and low-risk alpha types, the E6 and E7 proteins from low-risk types have a more limited effect on the cellular targets and pathways involved in the development of pre-cancer and cancer (Oh et al., 2004). Although the low-risk types, such as HPV-6 and HPV-11, do not have the same deregulation of viral gene expression as high-risk types, they can still be associated with anogenital warts and in rare cases, cancer (Egawa & Doorbar, 2017; Kovacic et al., 2006).

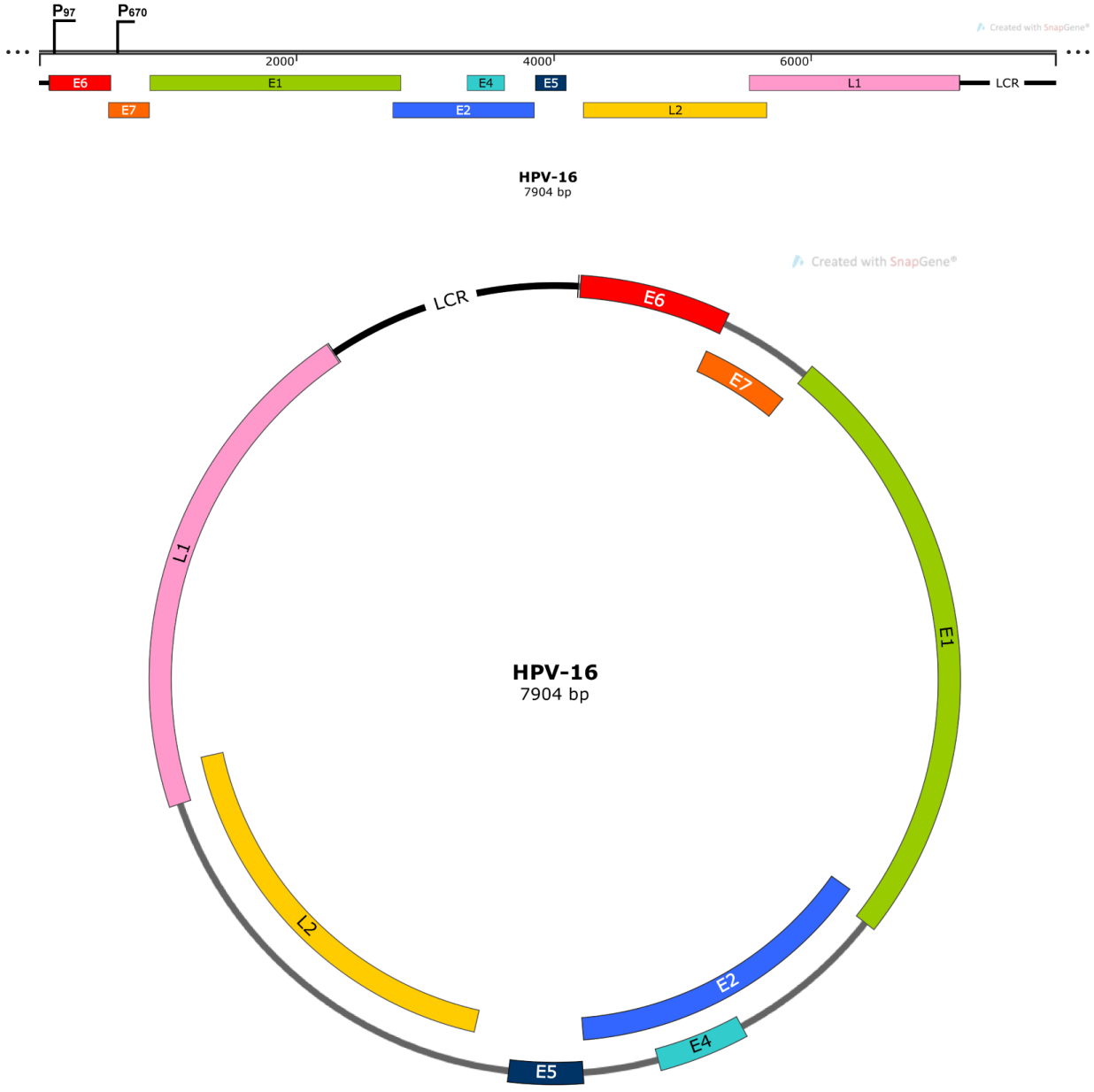


Figure 3a.- Genome organization of the high-risk (HPV16) Alpha-papillomavirus. The early (p97) and late (p670) promoters in HPV16 are shown in black sticks. Both low and high-risk genomes contain six or seven early (E) ORFs (E1, E2, E4 and E5), and E6 and E7 are expressed from the different promoters at different stages during epithelial cell differentiation. The late (L) ORFs of HPV16 (L1 and L2) are expressed from the p670 promoter in the upper epithelial layers as result of changes in splicing. The LCR or URR also contains the replication origin as well as post-transcriptional control sequences that contribute to viral gene expression.

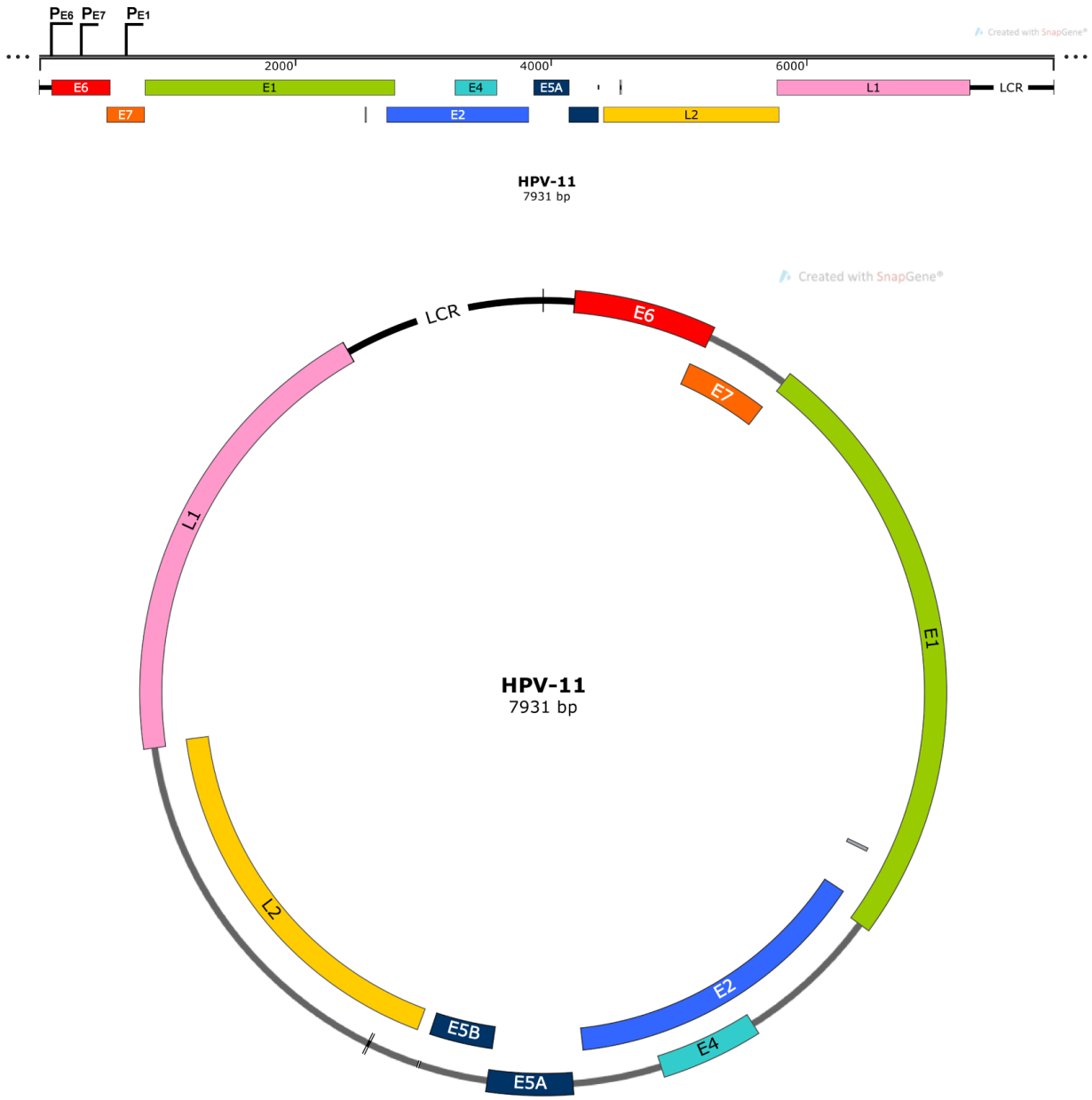


Figure 3b.- Genome organization of and low-risk (HPV11) Alpha-papillomavirus.

Core Genes		
Essential functions in viral genome replication and genome packaging		
Conserved between types		
Early proteins	E1	ATP-dependent helicase, role in papillomavirus genome replication
	E2	Coactivator of viral genome replication by recuitment of E6 to the viral replication origin
		Transcription factor of E6 and E7
		Important for viral genome segregation
	E4	Expressed as an E1-E4 fusion protein during the late phase of the virus life cycle
		Accumulated as cytoplasmic inclusion granules in Beta, Gamma and Mu HPV types
		Binds to cytokeratin filaments and disrupts cell structure
Late proteins	L1	Major capsid protein
		Assembles into pentomeric capsomeres which structure the icosahedral virion shell
	L2	Minor capsid protein, lso involved in encapsidation of viral DNA and in viral incoating after infection
Accesory genes		
Early proteins	E6	Differ between types (Table 3)
	E7	Modify the cellular environment to support and tolerate viral genome replication
	E5	Maximize the viral-fitness to complete viral life cycle in the site of infection

Table 2.- The function of core and accessory HPV proteins. Human papillomaviruses encode a group of core proteins that have conserved functions and therefore were vital in the early evolution of the virus. These include the early proteins E1, E2, and E4, and the late protein L2 and L1. The accessory proteins and early proteins E5, E6 and E7 have evolved through adaptation to different epithelial niches during the evolutionary history of HPV. The sequence and function of these genes are different between types and their main functions are to modify the cellular environment to support genome replication (Adapted from Doorbar et al. 2017).

HPV life cycle

The infection, replication and carcinogenic processes depend on the function of the viral proteins, where they are expressed in the epithelium, and whether this expression is properly controlled at a specific epithelial site. The use of organotypic raft cultures of spontaneously immortalized keratinocytes (NIKS) or immortalized human foreskin keratinocytes (HFKs) has allowed the reconstruction of the full productive life cycle of HPV in vitro (Meyers & Laimins, 1994). However, these models only permit analyses in the same epithelial-cell background, while HPVs infect various anatomical sites,

which have different epithelial backgrounds. Currently, there is almost no solid information about the viral life cycle at other sites of infection

Entry

Completion of the HPV productive life cycle depends on the nature of the epithelial site where infection occurs and on the presence of external factors such as hormones and cytokines (Gariglio et al., 2009). Infection requires an epithelial micro-wound to allow access of virus particles to the basal lamina, where they interact with heparin sulphate proteoglycans and possibly also laminin (Finnen et al., 2003; Johnson et al., 2009; Schiller et al., 2010). Structural changes in the virion capsid, including furin cleavage of L2, facilitate its transfer to a secondary receptor on the basal keratinocyte, which is necessary for virus internalization and transfer of the viral genome to the nucleus. Once internalized, the virions undergo endosomal transport, uncoating, and cellular sorting (Bugnon Valdano et al., 2019; Siddiqa et al., 2018). The L2 protein-DNA complex ensures the nuclear entry of the viral genomes, while the L1 protein is retained in the endosome where it undergoes lysosomal degradation (Bugnon Valdano et al., 2019; Schelhaas et al., 2012).

Infection

After entry, the wound healing process is necessary to stimulate the expansion of the infected cells. As a matter of fact, the formation of the initial lesion and the mitotically active cell division during wound healing is thought to be necessary for entry of the virus genome into the nucleus (Pyeon et al., 2009). Even though the proposed mechanism involves high-risk types, the route of infection may have multiple entry pathways depending on the virus type under study (Bousarghin et al., 2003; Day et al., 2003). It has also been hypothesized that the primary target cells for infection may be cells close to the squamo-columnar junction such as the epithelial reserve cells, which are located underneath the columnar epithelium of the endocervix (Doorbar, 2006), and which eventually form the stratified epithelial layers of the transformation zone as the cervix matures. Overall, the general hypothesis is that lesion formation begins with the infection of a basal stem cell, rather than a basal transiently amplifying cell, and that the longevity of the stem cells is a key factor in the formation of a

persistent lesion (Egawa, 2003; Schmitt et al., 1996). In the case of the low-risk HPV types that generally do not cause neoplasia, they do not stimulate basal cell proliferation greatly, but is still not known how this initial infection process relates to the disease outcome (Doorbar et al., 2012) (Figure 4).

Genome Amplification and Maintenance

It is thought that immediately following infection there is an initial phase of genome amplification and then maintenance of the viral episome at low copy number, independently of the nature of the infected basal cell (McBride, 2008, 2017; Parish et al., 2006). The proposed copy number in the basal layer of lesions is approximately 200 copies per cell, based on the study of episomal cell lines derived from cervical lesions. The basal copy number in benign oral papillomas has been quantified as 50 to 100 copies per cell, but it is likely that this varies from lesion to lesion and between different sites of infection (Maglennon et al., 2011).

The initial amplification phase requires the viral replication proteins E1 and E2 for episomal maintenance and replication, but they are dispensable once the copy number has stabilized (Angeletti et al., 2002; Kim & Lambert, 2002). The precise role of E1 and E2 in the epithelial basal layer during natural infections is still unclear. However, E2 may have a role in the regulation of accurate genome partitioning during basal cell division and in the regulation of viral transcription (McBride, 2008, 2013). The E2 protein has multiple binding sites in the viral long control region (LCR) and during viral DNA replication it recruits the E1 helicase to a specific E1 binding motif in the viral origin of replication. It has been suggested that the use of a viral DNA helicase that is different from the cellular replication helicases (MCM) allows the viral DNA replication to be disconnected from cellular DNA replication during genome establishment and amplification (Blakaj et al., 2009). However, the role of viral and cellular helicases in genome maintenance is still unclear.

After genome maintenance the amplification process occurs in the upper epithelial layers. The proliferation of the basal and parabasal cells is driven by high-risk HPV E6 and E7 proteins and it facilitates the expansion of the lesion size (Doorbar et al., 2012). The functional difference between the high- and low-risk E7 proteins is based on the differential ability to associate with members of the Retinoblastoma protein family (pRb). The high-risk E7 proteins bind and degrade both p105 and p107 proteins, which

control the cell cycle entry in the basal layer, and p130 protein, which is involved in cell cycle re-entry in the upper epithelial layer (Roman, 2006). The low-risk E7 proteins have a lower affinity for p105 and p107, but can associate with and degrade p130 in order to create an environment competent for replication and genome amplification in the mid-epithelial layers (Jenkins, 2007; Klingelhutz & Roman, 2012). Additionally, the high-risk E7 proteins stimulate host genome instability through deregulation of the centrosome cycle in the proliferating basal cells (Duensing et al., 2009; McLaughlin-Drubin & Münger, 2009).

Additionally, the high-risk E6s can upregulate telomerase activity to maintain telomere integrity during repeated cell divisions, and mediate the degradation of p53 within the cell (Galloway et al., 2005; Gewin & Galloway, 2001). The p53 function is inactivated by both high- and low-risk HPV type E6 proteins, but only the high-risk types and low-risk HPV-11 stimulate its ubiquitination and proteasomal-dependent degradation using the cellular ubiquitin ligase E6AP (Fu et al., 2010; Pim & Banks, 2010; Tomaić et al., 2009). Moreover, the high-risk E6 protein have the PDZ (PSD-95, Dlg, ZO-1) binding motif, absent in low-risk types, which allows the interaction with several PDZ proteins that are subject to degradation and are involved in the regulation of cell polarity, cell proliferation and cell signaling (Culp et al., 2006; Javier, 2008; Kühne et al., 2000). The main differences between low and high-risk types are show in Table 3.

Although E6 and E7 proteins cause neoplasia, their main function is not to promote basal cell proliferation, but to stimulate cell cycle re-entry in the mid-epithelial layers in order to allow genome amplification (Doorbar et al., 2012). Moreover, the expression of the E6 and E7 proteins in the upper epithelial layers promotes S-phase re-entry and thereby supporting viral genome copy-number increase. Additionally, the replication proteins E1 and E2 increase in abundance following the upregulation of the HPV ‘late’ or differentiation dependent-promoter (Bodily & Laimins, 2011). In HPV-16, this promoter, P670, resides within the E7 open reading frame. Thus, while the early promoter P97, located in the LCR, controls the transcription of E6 and E7 as the first and second ORFs, it appears that the P670 promoter upregulates the expression of E1 and E2 during differentiation to allow genome amplification (Doorbar et al., 2012; Romanczuk & Howley, 1992). This exposes the infected epithelial cell to differentiation signals and to the expression of differentiation markers, such as keratins 1 and 10 in cutaneous epithelium, or keratins 4 and 13 in mucosal epithelium; and at the same time, the expression of markers of cell cycle entry, MCM, ki-67, PCNA, CyclinE and

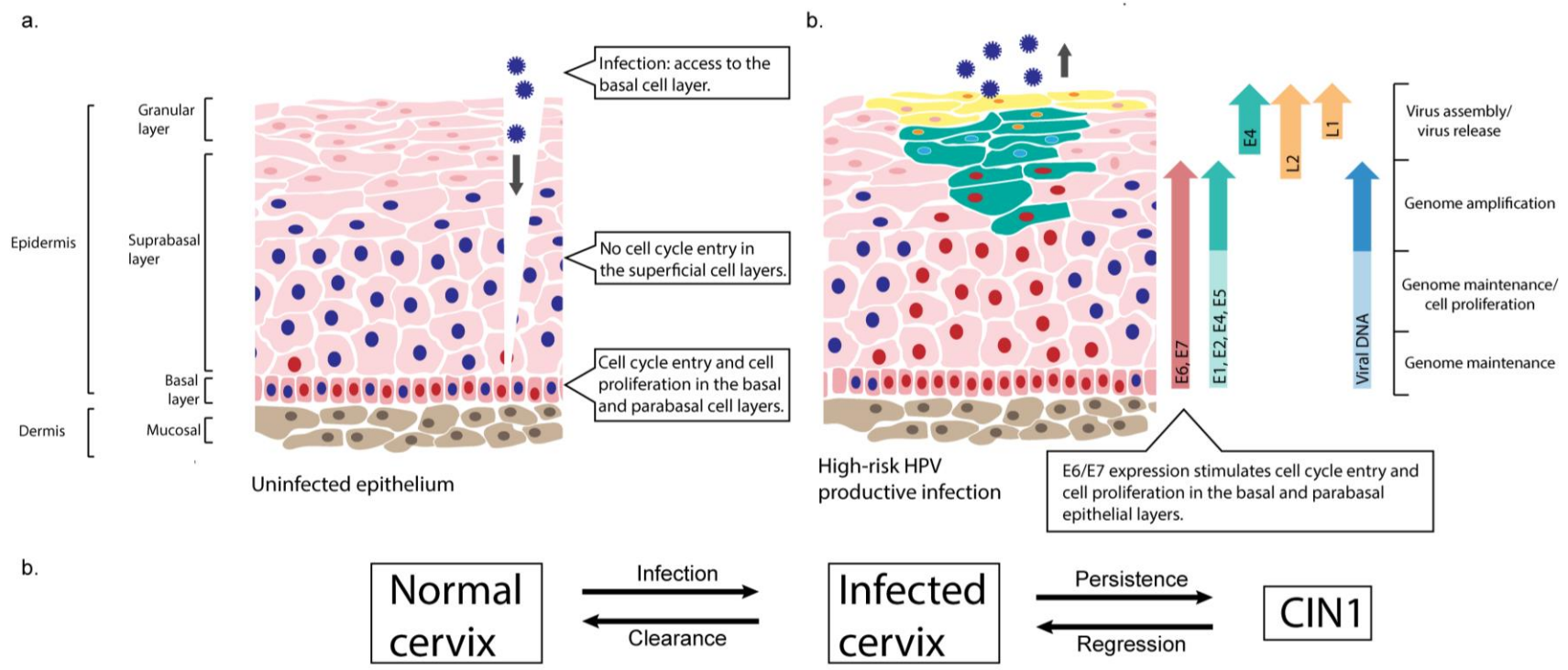
CyclinA (Doorbar et al., 2012). For the high-risk types, the S phase-like state marks the upper proliferative layers within the neoplasia, rather than a region where cell cycle re-entry has occurred. HPV genome amplification persists as the differentiating cell moves from an S-like phase to a G2-like phase, with viral genome amplification occurring primarily in G2 after cellular DNA replication has been completed (Banerjee et al., 2011; Wang et al., 2009).

In addition to E1 and E2, it is thought that the E4 and E5 proteins contribute indirectly to successful genome amplification, by modifying the cellular environment, with E5 also being thought to be responsible koilocyte formation (Krawczyk et al., 2008). E5 is a three-pass transmembrane protein with a cytoplasmic carboxyl-terminus, which may possess pore-forming capacity, and which interferes with apoptosis and the intracellular trafficking of endocytic vesicles (Krawczyk et al., 2008; Thomsen et al., 2000). E5 may also contribute to genome amplification through its ability to stabilize EGFR, to enhance EGF signaling and MAP Kinase activity, and to modulate both ERK1/2 and p38 independently of the EGFR (Crusius et al., 1998, 2000; Genther et al., 2003; Pim et al., 1992). The MAP Kinases ERK1/2 are critical modulators of nuclear E1 accumulation through the phosphorylation and activation of the E1 nuclear localization signal, and their activity is dependent on upstream MAPKs, MEK1/2 and p38 kinases. During the S and G2-like phases, Cyclins E and A, and their associated kinase cdk2, accumulate and contribute to the phosphorylation and inhibition of an E1 nuclear export sequence (Deng et al., 2004; Yu et al., 2007). This represents evidence that additional post-translational modifications in E1 might also facilitate differentiation-dependent genome amplification, and that the accumulation of E1 in the nucleus might enhance viral DNA replication, but not cellular replication, through induction of the DNA damage response (Moody et al., 2007).

Viral release and cycle completion

The E4 protein accumulates to very high levels in cells supporting virus particle synthesis and its primary function might be to promote virus release or transmission (Doorbar et al., 1991; McIntosh et al., 2008; Wang et al., 2004). In the case of HPV-16, E4 a growth arrest function has been shown to contribute towards amplification although not all HPV types share this activity.

The completion of the HPV life cycle is the packaging of viral genomes and virion release. This involves the expression of the minor coat protein L2, the exit of the cell from the cell cycle, and the production of the major coat protein L1, to allow the packaging of the genome. In HPV-16, a splicing change event, aided by high levels of E2, leads to the production of transcripts that initiate at the P670 promoter and terminate at the late polyadenylation site, instead of that in the early site (Doorbar et al., 2012). For the genome encapsidation, the L2 protein is recruited to the regions of replication before the expression of L1 and the assembly of the capsid in the nucleus (Day et al., 1998). The virus maturation occurs in the most superficial dying keratinocytes, which have converted from a reducing to an oxidizing environment just before virus release, allowing the progressive accumulation of disulfide bonds needed to stabilize the L1 protein interactions required for the production of infectious virions (Buck et al., 2005). The final assembled particles contain 360 molecules of L1 protein arranged into 72 pentameric capsomeres, with a smaller and variable number of L2 molecules (Buck et al., 2008).



b.

Normal cervix ⇄ (Infection / Clearance) Infected cervix ⇄ (Persistence / Regression) CIN1

Figure 4.- From infection to lesion formation. (a) The formation of the lesion is thought to be facilitated by micro wounds in the epithelium. (b) Regulation and deregulation of the life cycle is regulated during epithelial cell differentiation. Indeed, cells are driven through the cell cycle as a result of E6 and E7 expression (red nuclei). The up regulation of viral proteins E1 and E2, necessary for genome amplification requires activation of the viral late promoter in the upper epithelial layers (cells shown in green with red nuclei), with virus particles subsequently being released from the epithelial surface. (c) The high-risk HPV infection can lead to the persistence of viral genomes in the basal layer without the development of disease. Alternatively, a productive infection can be developed which eventually will lead to CIN1 in which viral gene expression is regulated as the infected cells differentiate. Clearance of infection and disease regression involves activation of a cell-mediated immune response and a suppression of viral gene expression. It is thought that viral genomes can persist in the basal epithelial cells with very limited gene expression, allowing possible reactivation under circumstances, such as immunosuppression (Maglennon et al., 2014; Maglennon et al., 2011). Redrawn from Doorbar et al. 2013. (Doorbar, 2013; Nakahara et al., 2005).

Accesory genes: differ between types			
Modify the cellular environment to support and tolerate viral genome replication Maximize the viral-fitness to complete viral life cycle in the site of infection			
	Function	High-risk	Low-risk
E6	Encodes E6* products	Yes	No
	Binding and degradation of p53	Yes	Weak binding and no degradation
	Binding to PDZ-domain proteins	Yes	No
	E6AP ubiquin ligase binding	Yes	Yes
	transcriptonal activator MAML1 binding	No	No
	Inhibition of p53 transactivation and acetylation	Yes	Yes
	Inhibition of Apoptosis	Yes	Unknown
	Growth arrest after DNA damage	Bypassed	Normal
	Inhibition of keratinocyte differentiation	Yes	Unknown
	Inhibition of interferon response	Yes	Weakly
	Activation of signaling pathways: AKT, WNT, NOTCH, mTORC1	Yes	Unknown
	Activation of Telomerase	Yes	No
	Activation of c-MYC	Yes	No
E7	Binding and degradation of: pRb, p107, p130	Yes	Weak binding and no degradation
	Binding and no degradation of: E2F1, Cullin2, HDAC	Yes	Weak binding to E2F1
	Binding of regulatory proteins: E2F6, p600, HAT, PP2A	Yes	Yes
	Induction of cell cycle entry and DNA synthesis	Yes	Yes
	Induction of genome instability	Yes	No
	Suppresion of STAT1 function	Yes	No
	Role in immortalization and transformacion	Yes	Unknown
	Signalling pathway modulation	AKT	Unknown
	Role in viral genome amplification	Yes	Yes

Table 3.- Differences between high-risk and low-risk E7 and E6 proteins.

Low-risk types life cycle and the association with cancer

In the case of recurrent respiratory papillomatosis (RRP) associated with HPV-11 , where laryngeal papillomas can persist for decades, regrow after surgical removal, and give rise to metastatic lesions ; it has been observed that the viral genome integrates into the host cell chromosome, suggesting deregulated viral gene expression followed by the acquisition of additional genetic and epigenetic modification as seen for the high-risk types . The rearranged HPV-11 genomes have also been observed in some of the RRP-associated cancers, but the patterns of viral gene expression have not been characterized. Compared to the prevalence of low-risk types, RRP is rare, suggesting it is most likely a multigene disease, in which there is a tissue-specific immune deficiency that prevents the effective clearance and control of HPV-6 or -11 infections, similar to the situation with Beta HPV types . Considering this, HPV-6 and -11 should be treated as high-risk types in this type of patients .

Individuals with Epiderma Verruciformis (EV) have an unusual susceptibility to Beta HPV infection , which is normally asymptomatic in general healthy population and which can produce visible papillomas in immunosuppressed people or HIV positive patients . EV patients are particularly prone to the development of squamous cell carcinoma caused by HPV5 and HPV8. Furthermore, several other Beta HPV types, including HPV 38 (Viarisio et al., 2011) are believed to be significant cause of non-melanoma skin cancer at sun exposed sites in immune suppressed individuals . Unlike the alpha HPV types development of skin cancer as a result of HPV infection is believed to follow a hit and run type of mechanism, where the virus initially enhances susceptibility to cancer causing mutations, which tumour subsequently developing which do not require continued presence of the virus .

In general, HPV infections are present everywhere in the skin of immunocompetent people without forming lesions, latent in an asymptomatic form, and the low-risk types are maintained and propagated in the general population as successfully as the oncogenic high-risk types . They infect long-lived basal epithelial cells, including stem or stem-like cells , or can modify the infected basal cell to extend its lifespan and confer stem-like characteristics. This allows HPVs to establish themselves in the lowest epithelial layers where mitotically active cells are found, and they persist in these cells as episomes . For both high and low-risk types, replication occurs once per cell cycle in undifferentiation/proliferation basal cell, where viral gene expression is limited to minimize the chance of immune detection .

Although the low-risk E6 and E7 proteins, do not influence basal cell division as high-risk proteins, they can modulate the basal cell layer density at particular stages of the life cycle, such as during lesion formation. Moreover, both of them limit the transition from basal to parabasal cell layers in order to maintain infection, and the differentiation process is essential in the establishment of productive infection and transmission of both .

As mentioned above, the conserved core genes of all HPVs have essential functions in viral genome replication and packing, but the accessory genes, E6 and E7, modify the cellular environment, performing similarly, but not necessarily having identical functions in supporting virus synthesis during the life cycles and virulence. Thus, one of the major differences between low and high-risk, is that low-risk types do not typically use their E6 and E7 proteins to drive extensive cell proliferation in the basal and parabasal cell layers, mainly because there is a different transcriptional regulation of E6 and E7 (Egawa & Doorbar, 2017; Gheit, 2019). Indeed, low-risk E6 and E7 proteins mediate cell cycle reentry to restore an environment competent for replication in the infected post-mitotic cells, but they do not drive cell division as high-risk types do. They also limit the differentiation of keratinocytes in the basal layer to retain a reservoir for infection and to drive differentiating cells into replication cells for genome amplification in the suprabasal layers (Doorbar et al., 2015; Gheit, 2019).

Indeed, Beta-HPV types are also an example of having a higher risk of cancer development in susceptible groups, including SCID (severe combined immunodeficiency) and EV patients, and have been studied in sporadic association with cancer. In the case of E6, instead of binding E6AP, it binds the MAML protein. MAML is a downstream component of the Notch signaling pathway, which controls the normal basal cell differentiation (Brimer et al., 2017; Meyers et al., 2013). Therefore, the MAML-induced regulation by Beta E6s, probably prevents the loss of the infected basal cell from the epithelial basal layer, which is the case of high-risk types, is achieved through p53 which regulates Notch levels indirectly through transcription . Unfortunately, the low-risk alpha types, are not understood at this levels, but it is known that their E6 proteins can modulate p53 function, albeit less dramatically, and their E7 proteins target the Rb family members, although they preferentially associated with p130, which regulates cell cycle entry in the upper epithelial layers, rather than p105 and p107, which control these processes in the basal and parabasal layers .

The pathogenic mechanism of the low-risk types is distinct from those of the high-risk types , and only a few studies have examined the possible functions of the low-risk E6 protein. In the case of HPV-11, E6 associates with E6AP in vivo, suggesting it could be able to target E6-associated protein for degradation . Indeed, Bak degradation through the low-risk HPV E6-E6AP complex has been observed . Furthermore, both low and high-risk E6 proteins bind p53 at its carboxy-terminal binding site, but this interaction does not induce p53 degradation by low-risk types , mainly because interaction with the p53 core domain it is necessary for high-risk degradation .

Moreover, it has been reported that HPV-11 E6 activates autophagy by inhibiting Akt/mTOR and Erk/mTOR signaling pathways. The cellular autophagic machinery captures intracellular pathogens in double-membrane vesicles for lysosomal degradation and has a crucial role in the host defense against viral infections . The canonical autophagy pathway is repressed by mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and its upstream AKT or mitogen-activated protein kinase (MAPK) signaling cascades through its phosphorylation of Unc-51-like kinase 1 (ULK1). This process prevents ULK1 translocation to the phagophore assembly site . Autophagy can be activated by the suppressive effect of AMPK and p53 on mTOR . In the case of HPV-11, it is possible that E6 ectopic expression may increase the energy requirement and trigger metabolic stress. The increased autophagy levels could maintain host cell survival and help virus proliferation and expansion. These results are in contrast with the observation of HPV-16 pseudovirion infection in HaCat cells or the ectopic expression of HPV-16 E6 in primary human foreskin keratinocytes, activate the PI3K/AKT/mTOR signaling, inhibiting autophagy . However, in the case of high-risk HPV, controversial information about activation or inhibition of autophagy has been reported .

High-risk HPV and cancer

The anogenital site is the most investigated site of HPV-induced malignancies. Almost all HPV-associated cancers arise at the cervical or anal transformation zones, where the stratified and columnar epithelia meet. It is not known yet why this region is susceptible to transformation by HPV, but it could be due to failure of the productive life cycle and deregulation of viral gene expression (Egawa et al., 2015; Wechsler et al., 2012). The cervical transformation zone contains specialized cells known as

reserve cells and a cuboidal cell at the squamo-columnar junction (Doorbar et al., 2012). Depending on the environment, these cells can differentiate into the stratified epithelium of the transformation zone or the columnar epithelium of the endocervix. The current model argues that productive viral life cycles are likely to be completed at the ectocervix, where there is a population of stem cells; while an incomplete infection is more likely to occur at the endocervix (Doorbar, 2005, 2018; Egawa et al., 2015). However, the pattern of viral gene expression may vary depending on the cell type, which might result in different outcomes (Mittal & Banks, 2017) (Figure 5).

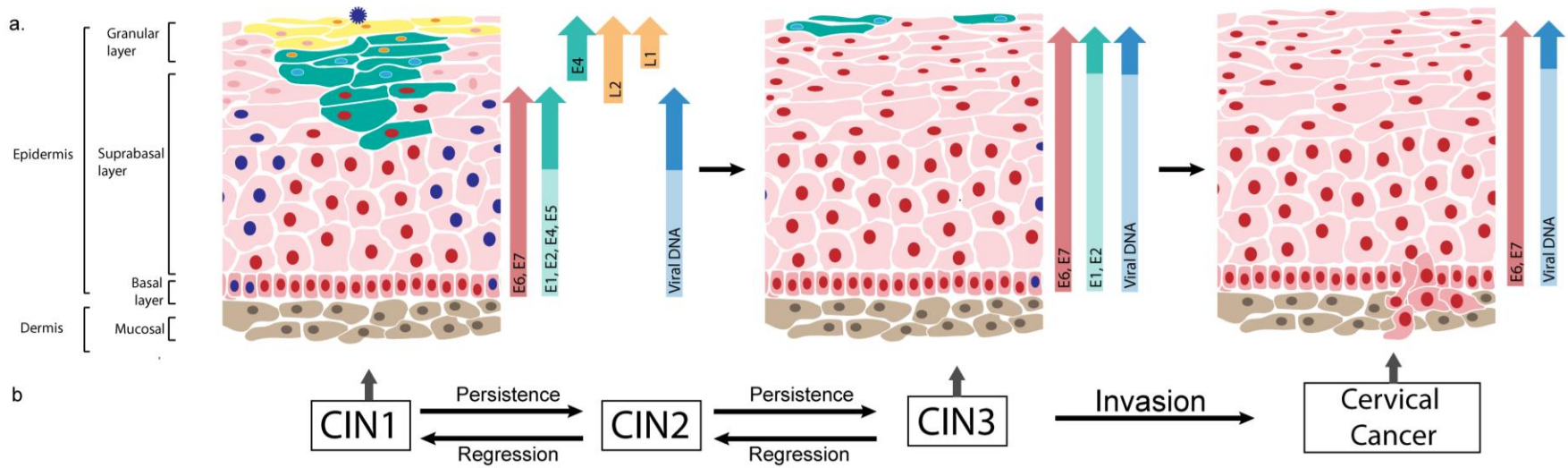

a.
Granular layer
Epidermis
Suprabasal layer
Basal layer
Dermis
Mucosal
E6, E7
E1, E2, E4, E5
E4
L2
L1
Viral DNA
E6, E7
E1, E2
Viral DNA
E6, E7
Viral DNA
b
CIN1
Persistence
Regression
CIN2
Persistence
Regression
CIN3
Invasion
Cervical Cancer

Figure 5.- To lesion persistence to cancer development. (a) In some instances, infection can lead to higher-grade neoplasia, with deregulated viral gene expression leading to genetic changes in the host cell and the integration of the viral genome into the cellular chromosome. The precancerous lesions seen in CIN2 and CIN3 have deregulated gene expression, which predisposes the cell to the development of cancer. Once a lesion has been established, the basal and parabasal epithelial cells can be driven into the cell cycle, either to mediate basal cell division or to drive cell cycle re-entry in the upper epithelial layers in order to support viral genome amplification. (b) In CIN3, late gene expression is retarded, and the production of infectious virions is restricted to smaller areas close to the epithelial surface. This situation is accompanied with elevated E6/E7 expression and represents a non-productive infection. Integration of HPV DNA into the host cell genome is facilitated by deregulated E6/E7 expression. In cervical cancer, high expression of the high-risk E6/E7 proteins in the basal layer leads to cell proliferation and invasion of the mucosal epithelium. In these cells, malignant transformation may develop. Redrawn from Doorbar et al. 2013.

The increase in the E6 and E7 expression levels correlates with progression of the neoplasia from CIN1 to CIN3 and is essential for maintenance of the transformed phenotype. E7 stimulates cell-cycle progression, partly through targeting the pRB pocket proteins (Boyer et al., 1996; Münger et al., 1989), while E6 inhibits apoptosis through the targeting of p53 (Scheffner et al., 1990) and Bak (Cornet et al., 2012; Thomas & Banks, 1998, 1999). Even though pRb and p53 are the main targets for driving transformation, other cellular targets are also important, and these have been suggested to vary depending on the virus type (Manzo-Merino et al., 2014; Pim et al., 2012).

The CIN1 lesions, corresponding to flat warts, usually maintain the ability to complete the HPV life cycle and produce viral particles, with a lower level of cell proliferation in the basal and parabasal layers (Middleton et al., 2003). In the case of CIN2+, there is an increase in E6 and E7 expression that generates an accumulation of genetic changes that contribute to cancer progression. This suggests that the low levels of E6 and E7 in CIN1 do not affect the functions of their cellular targets sufficiently to drive

cellular malignant transformation. Moreover, the viral deregulation observed in CIN2/3+ is thought to facilitate the integration of the viral episome into the host cell chromosome. It is believed that this integration process, besides driving the deregulation of E6 and E7 expression, can sometimes result in the disruption of viral genes that regulate the normal transcription from the LCR (Wentzensen et al., 2002). One of these genes is E2, which normally regulates E6 and E7 abundance by the release transcription of the integrated E6 and E7 genes from transcriptional inhibition . Most cervical cancers contain either one or many copies of HPV, integrated randomly into the host genome, with the viral integration site frequently lying within the regulatory E1 or E2 genes. Therefore, the integration and the loss of E6 and E7 regulation facilitates the constant high-level expression of these genes, with subsequent accumulation of genetic errors that lead to cancer. Around 70% of HPV-16-associated cervical cancers contain integrated HPV-16 sequences, while HPV-18 is almost always integrated in cancers. It is the long-term expression and over-expression of E6 and E7, and the resulting accumulation of genetic errors in the genome of the cell, that ultimately drives the progression from CIN3 to cervical cancer (Jeon et al., 1995).

The general hypothesis is that integration occurs in CIN2 and CIN3, which increases the deregulation of E6 and E7 expression (Häfner et al., 2008), driving an increase in cell division, promoting a lower sensitivity of the infected basal cells to cellular contact inhibition, and inhibiting the normal cellular differentiation program. Additionally, the E6 and E7 deregulation disrupts important regulatory pathways in the cell, mostly those mediated by the retinoblastoma protein family (pRB, p107 and p130), by p53, and by the PDZ-domain proteins, which control cell cycle entry, differentiation and cell polarity (Doorbar, 2006; Egawa et al., 2015).

The importance of E6 and E7 in maintaining the transformed phenotype is observed in cancer-derived cell lines that continue to express these oncoproteins many years after the primary immortalizing event. Numerous studies have reported that when these genes are knocked down by siRNA, cell growth is arrested and this leads to apoptosis (Butz et al., 2003; Chang et al., 2010; Cun et al., 2013; Hong et al., 2009; Jiang & Milner, 2002; Sima et al., 2008; Yamato et al., 2006). Additionally, E6 and E7 have complementary activities in immortalized primary human keratinocytes, since E6 interferes with cell survival pathways and E7 promotes cellular proliferation. When E6 or E7 are expressed alone, they do not have an impact on cellular immortalization.

Therefore, E6 and E7 are considered to be targets for therapeutic intervention and development of antiviral therapies.

HPV E6 oncoprotein

The HPV E6 protein from alpha-HPVs has approximately 150 amino acids, with two conserved zinc fingers formed by two pairs of CXXC motifs that are essential for the normal functioning of the protein (Barbosa & Wettstein, 1987; Cole & Danos, 1987) (Figure 6). Structural studies of the full-length E6 and the amino-terminal and carboxyl-terminal regions have confirmed the interaction of E6 with different cellular substrates (Martinez-Zapien et al., 2016; Nominé et al., 2006; Zanier et al., 2013). The principal target of high-risk E6 proteins is the tumour suppressor p53 (Scheffner et al., 1990, 1993). Normally, p53 is regulated by Mdm2, but upon DNA damage or viral infection, p53 is stabilized and activated by a series of phosphorylation pathways (Ashcroft et al., 1999; Kubbutat et al., 1997). In the presence of HPV, the Mdm2 pathway is inactive and p53 turnover is regulated by E6 in a proteasome-dependent manner. Additionally, E6 is involved in inhibiting p53 transcriptional activity, since E6 mutants unable to induce p53 degradation can still reduce p53 transcriptional transactivation activity. E6-mediated gene regulation of p53 is also achieved via the interactions with p300/CBP co-activators (Thomas & Chiang, 2005). Moreover E6-interacting regions of p300 are necessary for inhibition of p53-dependent chromatin transcription and E6-mediated repression of p53-dependent activation (Ashcroft et al., 1999; Pim et al., 1994; Thomas & Chiang, 2005; Zimmermann et al., 1999).

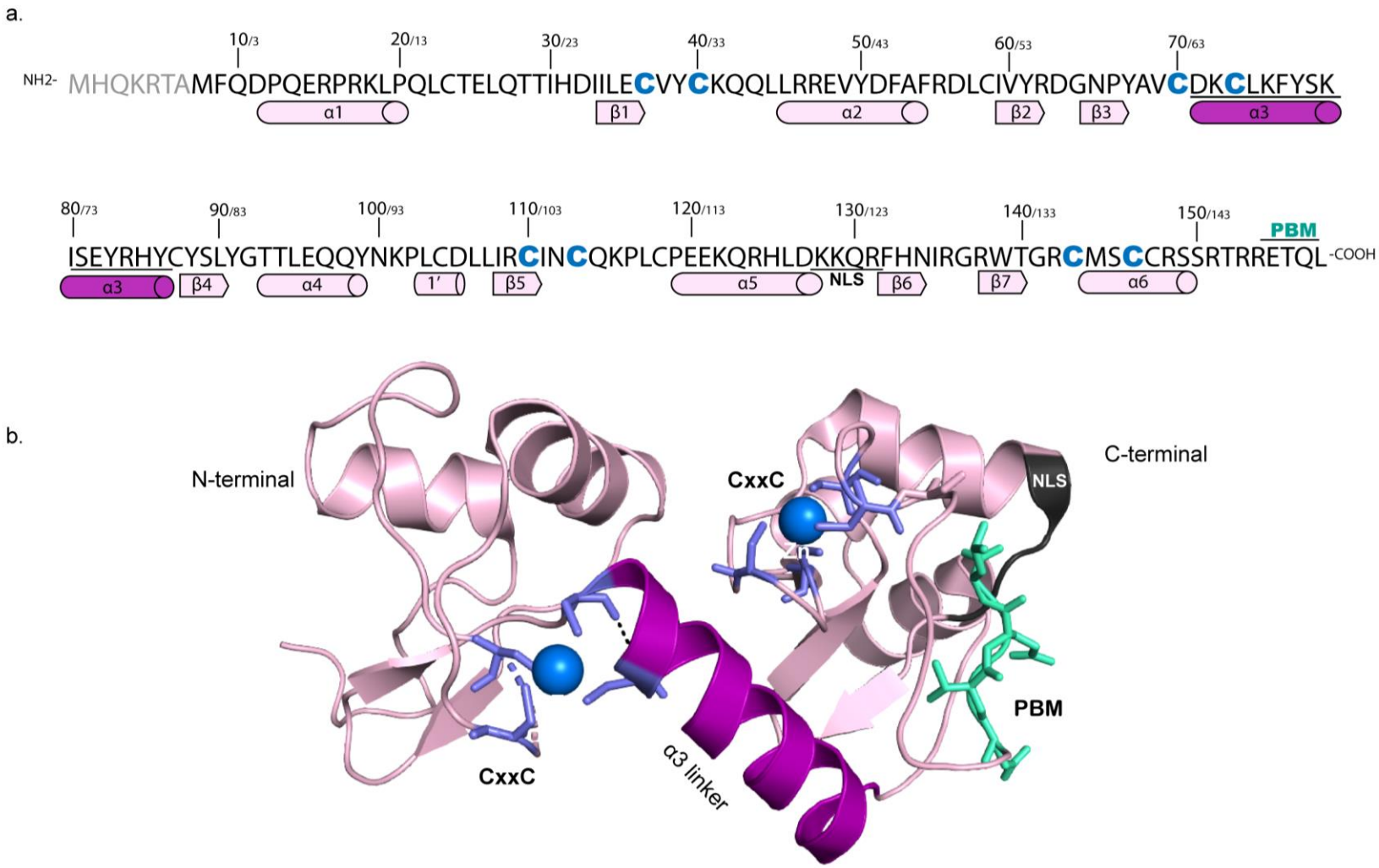

a.
10/3
20/13
30/23
40/33
50/43
60/53
70/63
NH2- MHQKRTAMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVYDFAFRDLCIVYRDGNPYAVCDKCLKFYSK
α1
β1
α2
β2
β3
α3
80/73
90/83
100/93
110/103
120/113
130/123
140/133
150/143
PBM
ISEYRHYCYSLYGTTLEQQYNKPLCDLLIRCINCQKPLCPEEKQRHLDKKQRFHNIRGRWTGRCMSCCRSSRTRRETQL-COOH
α3
β4
α4
1'
β5
α5
NLS
β6
β7
α6
b.
N-terminal
CxxC
NLS
C-terminal
PBM
α3 linker
CxxC

Figure 6.- HPV-16 E6 protein structure. The E6 protein can be numbered from the first methionine residue or the second (residue 8). Most structural analysis have been done counting the second methionine residue as the starting point, because prior residues lack a structured order. (a) The E6 protein from alpha-HPVs is composed of an amino-terminal domain (1 to 80 amino acids), an interdomain structured by a linker α3 helix (purple) and a carboxy-terminal domain, with a DNA binding-domain (Ristriani et al., 2000, 2001), a nuclear localization sequence (121-K-K-Q-R-124) (Le Roux & Moroianu, 2003), and a PDZ-binding motif (green). (b) The amino and carboxy-terminal domains are homologous and have two C-X-X-C motifs each (marked in blue), which associate in the tertiary structure and generate Zinc-binding sites (blue) important for E6AP binding. Both domains dimerize and form a hydrophobic pocket, where the LXXLL motif of E6AP is docked (Zanier et al., 2013). Mutations in residues of this hydrophobic pocket decrease LXXLL peptide binding and p53 degradation. Moreover, the carboxy-terminal domain has a DNA binding site in the vicinity of its zinc atom (β6, β7 sheets, α6 helix) (Ristriani et al., 2002). As expected for a nucleic acid binding domain, the HPV-16 carboxy-terminal domain displays a strongly positively charged surface, and the most positive surface potential is in E6 proteins from the two high-risk α9 and α7 species (Nominé et al., 2006).

E6 oncoprotein ubiquitination

In general, the degradative mechanism for other E6 proteins is less certain, but proteosome inhibitor studies have shown that the instability of E6, specifically HPV-16 and HPV-18 E6 proteins, is proteasome-dependent, with ubiquitination being the key element in targeting these proteins to the proteasome (Kehmeier et al., 2002).

The ubiquitin-proteasome system leads to the ubiquitination-dependent degradation of targeted protein by the proteasomal machinery. Before degradation, the substrate proteins must be tagged with ubiquitin moieties covalently attached by a sequential mechanism involving E1, E2 and E3 ligases, the latter being involved in binding the target proteins. The RING (really interesting new gene) E3 ubiquitin ligases catalyze the transfer of ubiquitin directly from E2 to the substrate, while HECT (homologous to

the E6AP carboxy terminus) E3 ligases accept the activated ubiquitin from E2 and then transfer it to the substrate. Moreover, the degradation is performed by the 26S proteasome, which contains two subcomplexes: the core 20S proteasome, which is a barrel-shaped structure; and the 19S regulatory particle, which contains approximately 20 different proteins and form the lid and base of the barrel (Lilienbaum, 2013).

The steady-state of high-risk E6 protein is increased when cells are treated with proteasome inhibitor MG132 (Jensen et al., 1995), while low-risk types were not stabilized by a proteasome inhibitor, and two cutaneous HPV E6 types (5 and 8) showed only slight stabilization.

Even though many studies have reported the importance of the E6/E6AP complex in E6 stability, there is no information available regarding the ubiquitination of E6 itself and the residues important for this process. Interestingly, it has been reported that HPV-16 E6 enhances the self-ubiquitination and degradation of E6AP; possibly by altering the conformation of E6AP, such that its catalytic domain has access to lysine residues elsewhere in the protein (Zanier et al., 2013). Additionally, the self-ubiquitination reaction appears to be the result of an intramolecular transfer of ubiquitin from the cysteine of the E6AP active site to one or more lysine residues of the same E6AP molecule. Whether one or more lysine residues of E6AP are targeted by multiple ubiquitin moieties, or a multiubiquitin chain is assembled at a single lysine is unknown. There is also evidence of intermolecular transfer of ubiquitin from one molecule of E6AP to another, suggesting that E6 mediates a dimer or multimer formation of E6AP. The E6AP self-ubiquitination is also stimulated by HPV-18, HPV-33 and HPV-39 E6 proteins, suggesting it is probably a cancer-causing trait (Kao et al., 2000). These results indicate that the ubiquitin-proteasome system is a key post-translational mechanism used by HPV to regulate the intracellular levels of its own gene products and their targets. Although it has been demonstrated that the degradative process of E6 does not require interaction with E6AP (Huibregtse et al., 1993; Huibregtse et al., 1995), it is still not clear which protein ligase ubiquitinates E6 for further degradation.

E6AP also stabilizes E6 from HPV-16 and HPV-18 (Tomaić et al., 2009). In the absence of E6AP, E6 protein levels are low, and it is rapidly degraded at the proteasome. Thus, E6AP is required for most E6's biochemical activities, with loss of E6AP mimicking E6 ablation (Kelley et al., 2005). Whilst E6 needs E6AP for its stability, its functions are not necessarily E6AP-dependent with respect to substrate targeting and degradation, since catalytically inactive E6AP is also capable of

stabilizing E6 (Tomaić et al., 2009). This demonstrates that HPV-16 and HPV-18 E6 proteins are regulated by the proteasome independently of E6AP, suggesting that other ubiquitin ligases may be involved in regulating E6 turnover. Furthermore, E6 can degrade many of its substrate proteins, although not p53, in the absence of functional E6AP, suggesting the existence of additional degradation routes (Camus et al., 2007; Massimi et al., 2008; Vats et al., 2019).

E6-induced perturbation of the ubiquitination system

The ability of E6 proteins to bind E6AP is important for modifying and promoting the proteasomal degradation of their partners. E6 protein acts as a bridge between E6AP and its substrates, resulting in polyubiquitination of the target protein and degradation at the 26S proteasome (Scheffner et al., 1993).

Most of the studies have focused on the high-risk types, since E6AP is necessary for the oncogenic activity of E6, but the interaction with E6AP has also been observed with low-risk mucosal HPVs, which also interact through the LXXLL motif on E6AP (Brimer et al., 2007; Chen et al., 1998; Kuballa et al., 2007; Scheffner et al., 1993); and has been extended to certain cutaneous HPVs of the beta genus (Bedard et al., 2008; Underbrink et al., 2008). However, these interactions are not as conserved as those of the alpha group, since several beta type E6 proteins do not bind E6AP (White et al., 2012). The possible general role for the E6-E6AP complex has been based on the common targeting of Bak by nearly all tested E6 proteins (Thomas & Banks, 1998, 1999; Jackson et al, 2000; Bedard et al., 2008; Underbrink et al., 2008; Westphal et al., 2011). Additionally, the alpha genus E6 proteins associate preferentially with E6AP by binding to the LXXLL peptide on E6AP and stimulating E6AP ubiquitin ligase activity, while the E6 proteins from the beta, gamma and delta genera bind to a similar LXXLL peptide on the cellular transcriptional co-activator MAML1, repressing Notch signaling. None of the E6 proteins studied so far have been observed to interact physically with both E6AP and MAML1, suggesting that this divergence of E6 protein functionality might have been a major event in papillomavirus evolution (Brimer et al., 2017; Drews et al., 2020).

Depending upon the HPV type, the affinity for E6AP can differ. HPV-16 and -31 E6s can target both the wild-type and a T485E phospho-mimic mutant of E6AP for degradation, while, in contrast, HPV-18 E6 can target the wild-type E6AP, but not the

T485A mutant, being unable to stimulate highly active auto-degradatory activity. E6AP phosphorylation on T485 not only regulates its enzymatic activity, but also modulates its subcellular distribution, and this process is completely overridden by E6. It has also been shown that this E6-mediated nuclear accumulation of phospho-E6AP is partly dependent upon the activity of 14-3-3 proteins, suggesting that the recruitment of phospho-E6AP to the nucleus requires the E6's ability to recognize 14-3-3 proteins. From the point of view of viral evolution, it is possible to speculate that E6 has evolved the ability to recruit phospho-E6AP to the nucleus to overcome possible E6 instability caused by the lack of active E6AP during the cell cycle or differentiation phase, when E6AP becomes highly phosphorylated. Thus, E6 has evolved to efficiently overcome the negative regulation of E6AP activity; how this varies between HPV types needs to be further investigated (Thatte & Banks, 2017).

Besides E6AP, several ligases from the ubiquitination system have been identified as partners of E6. Two ligases of the HECT-domain type have been identified: of these, HERC2 is a putative E3 ligase that has been associated with HPV-16 E6 (Martinez-Noel et al., 2012; Martínez-Noël et al., 2018; Vos et al., 2009); and EDD has been shown to bind strongly to HPV-18 and weakly to HPV-6 and HPV-11 E6 proteins (Tomaic et al., 2011). None of these have been tested for the ability to ubiquitinate E6, but it is assumed that they are recruited by E6 to affect other substrates. For instance, EDD enhances ubiquitination and therefore reduces E6AP, reducing E6 levels indirectly. Additionally, HERC2 stimulates the ligase activity of E6AP, possibly enhancing the activity of the E6-E6AP complex, instead of simply targeting E6 itself (Kühnle et al., 2011). Moreover, many proteomic studies have identified different proteasome subunits as E6 targets (Rozenblatt-Rosen et al., 2012; Tomaic et al., 2011). The S5a subunit binds strongly to E6 in an E6AP-dependent manner and is subjected to increased levels of ubiquitination in the presence of E6, both in vitro and in vivo. Furthermore, in vitro assays have reported S4 and S8 subunits as being the strongest direct interacting partners of HPV E6, while weak interaction was observed with the S2, S6a, S6b and S7 subunits, and no interaction was observed with S5a or S9 in vitro (Tomaić et al., 2013).

Other HPV oncoproteins have also been reported to interact with different members of the ubiquitin proteasome system. HPV-38 E6 interacts with the ubiquitin protein ligase E3 UBR4/p600, which is a common interactor with the E7 proteins of HPV-16, -6b, -11 and BPV-1 (DeMasi et al., 2005; Huh et al., 2005). It has also been reported

that HPV-16 E6 binds USP15, a deubiquitinating enzyme. Both HPV-16 and HPV-18 E6 proteins are stabilized by catalytically active USP15, but not by the inactive mutant (Vos et al., 2009). This suggests that USP15 might protect E6 by removing the ubiquitin chains.

E6 can also interact with other modifying proteins that are not involved in ubiquitination. The HPV-16 E6 protein interacts with the p300 acetyltransferase, inhibiting the acetylation, and therefore the activation, of p53 (Xie et al., 2014). HPV-8 E6 protein also specifically interacts with p300 acetyltransferase and enhances its degradation (Howie et al., 2011).

E6 oncoprotein phosphorylation and phospho-regulation

Besides the well-studied interaction with the ubiquitin system, E6 has also been studied as a phosphoprotein. One of the first examples was the short form of cottontail rabbit papillomavirus E6 oncoprotein, which can be phosphorylated on tyrosine, threonine and mostly on serine residues, although the specific residues and the kinase involved were not identified (Barbosa & Wettstein, 1987). Later, two-hybrid system experiments showed that high-risk E6 proteins interact with protein kinase PKN, a fatty acid and Rho-small G protein-activated serine/threonine kinase, significantly homologous to PKC (Gao et al., 2000; Palmer et al., 1995). This interaction was verified by in vitro GST binding assays, in which the full-length PKN substantially bound to in vitro translated HPV-16 and -18 E6 proteins. Additionally, they showed however, that HPV-16 E6 does not target PKN for degradation, but instead that HPV-16 E6 is phosphorylated by PKN in vivo (Gao et al., 2000). Although, the role of PKN phosphorylation in E6 transforming activity was not defined, it was observed that E6 binds to the carboxy-terminal region of PKN, suggesting the possibility that Rho and E6 may concurrently interact with PKN, potentially allowing E6 to influence Rho-mediated signaling, and linking the phosphorylation of E6 with E6-induced immortalization or the regulation of viral life cycle.

Subsequent studies demonstrated the involvement of the conserved carboxy-terminal PKA consensus site, in regulation of the PDZ-binding domain of HPV-18 E6 (Kühne et al., 2000). It was reported that GST-18E6 could be phosphorylated in the presence of NIH3T3 cell extracts, and that this activity was reduced in the presence of PKA-inhibitor peptide (PK-i); showing that activation of the PKA pathway leads to an

increase in the level of E6 phosphorylation. Furthermore, the Thr156Val substitution showed that this was the phospho-acceptor site for PKA in vitro (Figure 7). Additionally, the induction of PKA leads to a stabilization of the DLG protein in HPV-positive cells. Thus, it was shown that E6-Dlg interaction is inhibited by PKA phosphorylation on Thr156, and that HPV-18 E6-induced degradation of DLG was also regulated by PKA phosphorylation in vivo. This demonstrated that the ability of E6 to recognize the PDZ proteins is not constitutive, since the phosphorylation of the threonine residue within this site inhibits the binding with PDZ proteins. It also showed that the PKA consensus recognition site is very highly conserved in all high-risk types; linking the PKA phospho-regulation of E6 with the levels of cAMP and the malignant progression of HPV-containing cells (Kühne et al., 2000).

Subsequently, *in vitro* assays also demonstrated that HPV-16 E6, but not HPV-11 E6, was a substrate of PKA, confirming that PKA phosphorylation is specific to high-risk HPV types (Boon et al., 2015; Boon & Banks, 2013; Delury et al., 2013a; Kühne et al., 2000). Furthermore, within the high-risk types, the level of phosphorylation differs depending on the genotype. I*n vitro* phosphorylation experiments with equal amounts of the purified E6 proteins, fused to GST, have helped in determining whether the E6 proteins from different genotypes can be considered to be “weak” or “strong” substrates for any given kinase. Thus, HPV-18 E6 is the strongest PKA substrate, followed by HPV-16 E6 and HPV-31 E6, the latter being much weaker. Other kinases were also assessed but showed little or no phosphorylation of E6. In the case of CamKII, no phosphorylation was observed; and very weak non-Thr156-dependent phosphorylation was observed with PAK, suggesting that there are other phosphorylation sites on E6. In the case of AKT, HPV-16 E6 is the best AKT substrate, with HPV-31 and HPV-18 E6 being only weakly phosphorylated. However, the level of AKT phosphorylation on HPV-18 E6 Thr156 was significant, and a second weaker site in HPV-18 E6 most likely also exists (Figure 7). Interestingly, *in vivo* experiments with transiently transfected HPV-16 E6 and HPV-18 E6 showed that, in the absence of the PKA stimulator forskolin, HPV-18 E6 appears to be only weakly phosphorylated, while there was a slightly higher level of phosphorylation of HPV-16 E6. However, when forskolin was added, it resulted in dramatic increases in the levels of phosphorylation of both HPV-16 and HPV-18 E6, suggesting that phosphorylation of E6 is condition-specific and varies between high-risk E6 proteins (Boon & Banks, 2013).

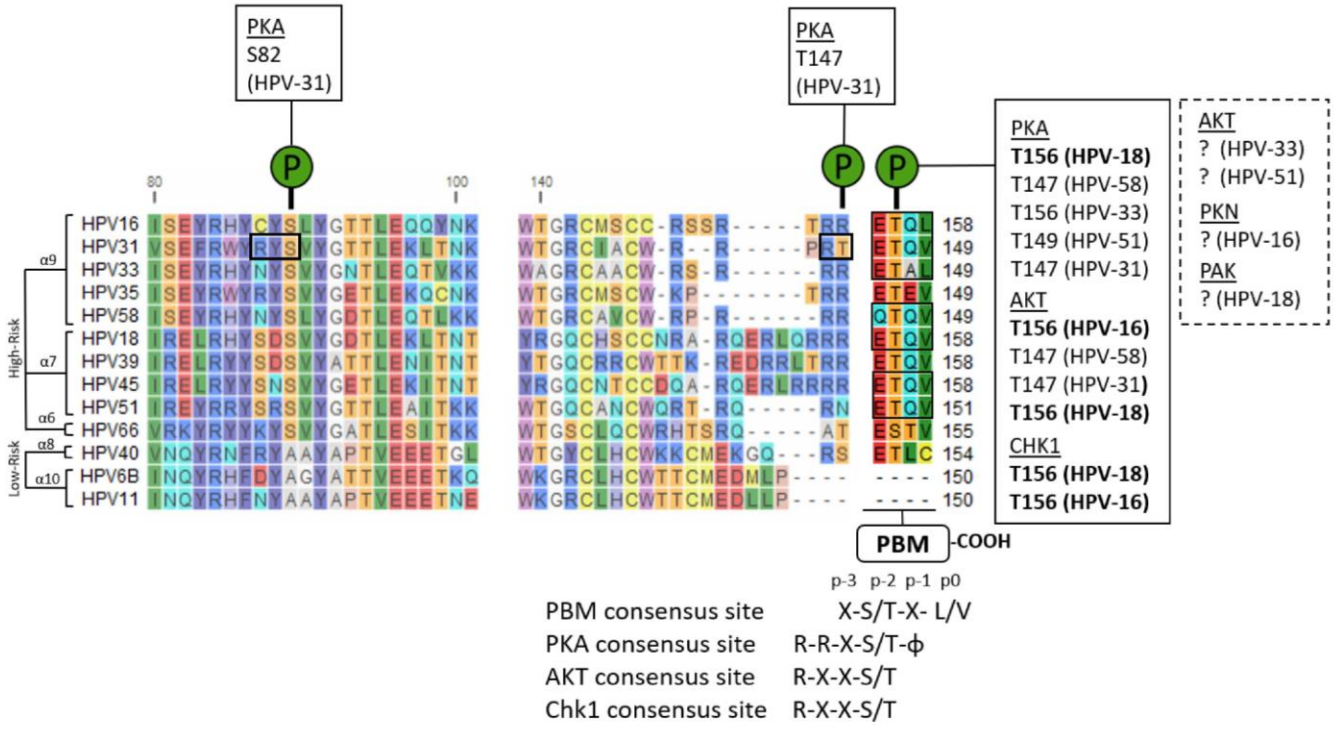


Figure 7.- Phosphorylation of E6. Alignment of relevant regions of the HPV E6 protein from genotypes from the high- and low-risk clades. Genotypes which have been evaluated in phosphorylation assays are represented. Most high-risk types evaluated are phosphorylated in the threonine residue in position -2. The genotypes marked in bold are those highly phosphorylated for the given kinases.

The PBM/PKA module has dual functions, depending on the phosphorylation status of the Thr156 residue, which confers either recognition of PDZ proteins or recognition of the members of the 14-4-3 protein family, which do not seem to be a standard degradation target of E6 (Boon & Banks, 2013; Thatte et al., 2018). These are phospho-threonine/serine-binding proteins implicated in the regulation of many different cellular processes, including those directly relevant for cancer progression and malignancy (Hong et al., 2010; Muslin et al., 1996; Neal & Yu, 2010). High-risk HPV E6s can recognize 14-3-3ζ in a PKA or AKT phosphorylation-dependent manner and this interaction helps to maintain steady-state levels of the HPV-18 E6 protein (Boon & Banks, 2013). In the absence of phosphorylation, 14-3-3ζ does not interact with HPV-18 E6. After phosphorylation, the interaction with DLG and MAGI-1 decreases, while there is a corresponding increase in interaction with 14-3-3ζ.

Similarly, there is a phospho-dependent interaction between HPV-16 E6 and 14-3-3ζ, but not as strong as that of HPV-18 E6. Additionally, HPV-16 and HPV-31 E6 proteins are phosphorylated by AKT and promote interaction with 14-3-3ζ, but AKT phosphorylation of HPV-18 E6 is not sufficient to confer a significant degree of association with 14-3-3ζ, as PKA phosphorylation does. This demonstrates that high-risk E6s interact with 14-3-3ζ in a conserved manner, and that the differences are determined by the identity of the kinase recognizing the E6 phospho-acceptor site, and, thus, are ultimately determined by the differences in the PBM sequence (Basukala et al., 2020; Boon & Banks, 2013).

Subsequent studies with other high-risk HPV E6s confirmed that the different efficiencies in association with 14-3-3 proteins, depend exclusively on the carboxy-terminal sequence of the PBM (Boon et al., 2015; Thatte et al., 2018). It was observed that, after HPV-18 E6, the most susceptible to PKA phosphorylation was HPV-58, and to a weaker extent HPV-33, -51 and -31 E6s. In the case of AKT, HPV-16 and HPV-58 E6s are the most readily phosphorylated, followed by HPV-31 and HPV-33, with HPV-51 E6 being a very poor substrate for AKT. Overall, lower levels of phosphorylation were found on HPV-33 and -51 E6, but their phospho-acceptor sites are still unknown (Boon et al., 2015). Furthermore, some residues upstream of the PBM can also be phosphorylated. In the HPV-31 E6 carboxy-terminal sequence there are two threonine residues: one within the PBM (Thr147) and one upstream of it (Thr145). Phosphorylation of T145 is unlikely to affect PDZ recognition, while T147 is inhibitory (Boon et al., 2015). It was shown that these two residues, when mutated, had no effect on PKA phosphorylation, indicating that the PKA phospho-acceptor site is more likely to be Ser82. However, the S82A mutant can still be phosphorylated by PKA, further indicating that multiple phosphorylation sites for PKA are present on HPV-31 E6. In contrast, AKT phosphorylation occurs primarily on the T147 within the PBM. Consistent with this, phospho-specific antibodies, which recognize PKA-phosphorylated HPV-18E6 and HPV-58 E6, cannot recognize PKA-phosphorylated HPV-31 E6, confirming again that phosphorylation occurs outside the PBM. Moreover, the AKT-phosphorylated HPV-31 E6 is recognized by the phospho-specific antibody; therefore, HPV-31 E6 can be phosphorylated by both AKT and PKA, but the phospho-acceptor sites are not the same and only AKT phosphorylates within the PBM (Boon et al., 2015).

The consequences of phosphorylation for PDZ recognition are similar between the different E6 proteins, since phosphorylation of the PBM, even if weak, leads to a reduction in PDZ binding potential (Boon et al., 2015; Boon & Banks, 2013; Thatte et al., 2018).

In order to assess which other kinases could be phosphorylating E6, it is necessary to know when and why the E6 protein is modified. It was shown that HPV-18 E6 is only very weakly phosphorylated or is unphosphorylated during the normal cell cycle. However, during induction of stress-responses kinases, particularly those associated with the DNA damage response, there is a strong increase in the levels of HPV-18 E6 PBM phosphorylation, which appears to be mainly mediated by Chk1 kinase, demonstrating a link between the regulation of the E6 PBM activity and the induction of the DNA damage response (DDR) (Thatte et al., 2018). Experimentally, it was demonstrated that E6 can be phosphorylated by a variety of stress response kinases, since the induction of an oxidative-stress response by peroxide or nocodazole was responsible for very high levels of E6 phosphorylation, as well as Cycloheximide treatment, but the latter seems to be mediated by a different stress response pathway (Thatte et al., 2018). In vivo, the situation is different, since the kinase responsible for phosphorylation depends on the specific stimulus used for DDR. Both peroxide and nocodazole induced phosphorylation of E6 through the oxidative stress pathway, as was demonstrated by blocking this pathway with NAC (N-acetylcysteine). In the case of nocodazole, Chk1 phosphorylates E6; however, in contrast with the experiments in vitro, Chk2 is involved in phosphorylation of E6 when cells are treated with peroxide, but through the activation of PKA and not by direct phosphorylation, as was demonstrated using the H89 inhibitor (Figure 8). Furthermore, the phospho-regulation of high-risk HPV E6 oncoproteins by the induction of oxidative stress and DDR can perturb p53 transcriptional activity on a subset of p53-responsive promoters (Thatte et al., 2018).

Additionally, E6 promiscuity appears to have co-evolved with the consensus phosphorylation site, since the residues conferring phosphorylation by PKA or CHK1 are also critical for interaction with DLG, MAGI-1 and SCRIB in the case of the cancer-causing types (Sarabia-Vega & Banks, 2019). All this information points to the importance of phosphorylation, and possibly other post-translational modifications, in the carcinogenicity of E6 proteins. Discovering which PTMs are crucial for virus stability and progression to cancer might open a window for therapeutic approaches.

It is clear that the initial appearance of the ancestral PBM probably gave the virus and advantage in colonizing new niches, where it would probably have bound only a restricted number of PDZ targets. Subsequent evolution of the PBM may in some cases have increased its functional flexibility with respect to the numbers of targets that could be bound, but also increased the risk of inducing changes that lead to malignancy. This explains why certain HPV types have PBMs but are not associated with cancer. Furthermore, we do not yet know if the capacity of the PBM to be phosphorylated is a trait gained after niche adaptation which confers more flexibility upon already carcinogenic types, or if it is a trait HPVs gained before becoming carcinogenic.

Therapeutic implications of targeting E6 phosphorylation

As has been described, the E6 protein of high-risk types associated with cancer has been shown to be phosphorylated by primarily three kinases. While phosphorylation of E6 by Chk1 and PKA obviously has major effects upon of HPV E6 function, particularly in the context of a DDR, not much information about the effect of AKT-phosphorylation of E6 has been reported.

Indeed, commonly used chemotherapeutic compounds induce high levels of E6 phosphorylation by Chk1 and PKA within the PBM, resulting in increased association with 14-3-3 proteins and subsequent inhibition of p53 transcriptional activity. This suggests that this regulatory mechanism has evolved in the oncogenic E6 proteins as an additional means of overcoming the DDR and p53 transcriptional activation. Whilst the biological relevance of this *in vivo* has yet to be defined it is very tempting to speculate that inhibitors of Chk1 signaling in the context of HPV induced malignancy might also offer increased therapeutic potential when used in combination with standard chemotherapeutic regimes, possibly accompanied by the use of CKII inhibitors for blocking E7 protein phosphorylation. Current studies are assessing these combination therapies in various experimental settings.

Cancer cells are characterized by unstable genomes and activation of DNA repair pathways and radiotherapy and chemotherapy cause DNA damage. Cancer cells can be made more sensitive to the effects of radiation, which is radiosensitization, through inhibition of DNA repair pathways. The ATM/Chk2 and ATR/Chk1 pathways are principal regulators of cell cycle arrest, following DNA DSB or SSB and Chk1

determines the cellular response to DNA damage and governs G1/S, S and G2/M phase checkpoints (Carrassa & Damia, 2017; Yang et al., 2016). It has been observed that Chk1 was found to be overexpressed in several human tumours, including colon, liver, breast, and gastric carcinoma, promoting growth. Its expression often positively associates with tumour grade and disease recurrence (Yang et al., 2016). In cervical cancer, Chk1 and phopho-Chk1 were visibly elevated and correlated with poor prognostic factors; and was gradually increased during cervical lesion progression and this positively correlated with HPV-16 E6 and E7 mRNA. In contrast, knockdown of Chk1 suppressed cervical cancer cell proliferation and induced cell cycle arrest during S phase, confirming that increased Chk1 expression possesses survival advantages in cervical cancer and correlates with HPV16 E6/E7 expression (Lin & Chen, 2018). Since the E6 protein is also phosphorylated by Chk1 and Chk2 in the presence of DNA damage inducing agents, inhibitors for these two kinases may be of value for treatment of cervical cancer. Several Chk1/Chk2 inhibitors have been studied from which PF-00477737 (ClinicalTrials.gov: NCT00437203); LY2603618 and MK-8776 (SCH 900776) have proven to be potent and selective and cause chemosensitization with gemcitabine. They are currently used in phase I and phase II clinical trials (Figure 8) . Obviously, additional studies are required to fully understand the relevance of DNA damage induced phosphorylation of E6 during tumour progression.

Additionally, the cAMP/protein kinase A (PKA) signal pathway has been studied thoroughly and it has been shown to regulate cell proliferation, differentiation and apoptosis of cancer cells (Tortora & Ciardiello, 2002a, 2002b). One of the first inhibitors and most promising to be tested as a drug was 8-Cl-cAMP (Figure 8), which is an analog of cAMP, which induces growth inhibition in human carcinomas (Cho-Chung & Nesterova, 2005; Schwede et al., 2000). It has been evaluated in phase I/II clinical trials as an anticancer agent . Moreover, the inhibition of PKA has a cooperative antitumor effect with a selected class of cytotoxic drugs and radiotherapy . The PKA inhibitor H-89 (Figure 8), could modulate the over-expression of DNA damage repair and antiapoptotic proteins in the radioresistant cells; combined therapies have shown that H-89 can reduce the levels of Bcl-2 and enhanced p53 in radioresistant cells, potentiating cytotoxicity of radiation and working as a radiosensitizer (Chin et al., 2005).

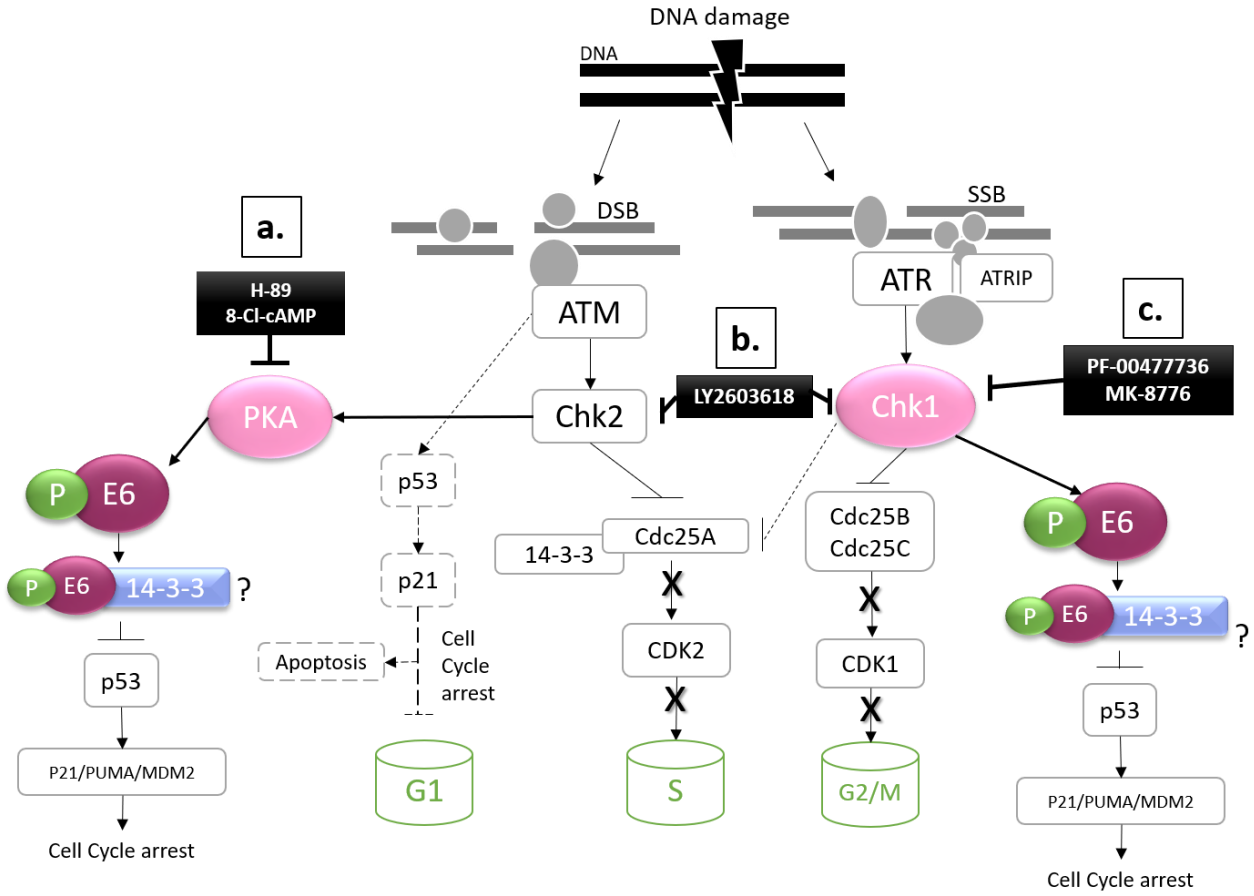


Figure 8.- DNA repair pathways in cancer cells. The ATM/CHK2 and ATR/CHK1 pathways are activated by DNA double-strand breaks (DSB) or by DNA single-strand breaks (SSB) and replication stress, respectively. Cell cycle checkpoints are induced primarily through p53, CHK2, CHK1 and p38/MK2, which are phosphorylated by ATM and ATR. Activated p53 leads to G1-phase arrest and induces apoptosis. Activated CHK2 and CHK1 promote the phosphorylation of CDC25 proteins, which are further sequestered by 14-3-3 proteins and rapidly degraded. This abolishes the activation of Cdk1 and Cdk2, thus stopping cell cycle progression either in S-phase or at the boundary of G2/M resulting in cell cycle arrest. Cancer cells deficient in p53 due to mutation or deletion lack the G1 checkpoint and are more dependent on the intra-S and G2/M checkpoints. HPV E6 protein is phosphorylated directly by Chk1 and PKA, the latter is firstly activated by Chk2 in the context of DDR, which promotes interaction with 14-3-3 proteins and inhibition of p53. The different protein kinase inhibitors currently used in clinical trials are indicated: H-89 and 8-Cl-cAMP for PKA (a), the dual Chk1

Chk2 inhibitor LY2603618 (b), and the Chk1 inhibitors PF-00477736 and MK-8776 (c). SSB: Single-strand break; DSB: Double-strand break. ATR: Ataxia-telangiectasia and Rad3 related; ATRIP: ATR-interacting protein. ATM: Ataxia-telangiectasia mutated.

PDZ domains and PDZ-binding motifs

The PDZ proteins have the most widely distributed protein-protein interaction domains in metazoans and are widely used to assemble signaling complexes (Kim et al., 2012). The PDZ domain was first recognized in the proteins post-synaptic density-95, discs large, and zonula occludens 1 (Cho et al., 1992; Kim et al., 1995; Woods & Bryant, 1993) as a common component of such scaffold proteins; in fact the term PDZ is an abbreviation for the first three proteins found to share this structural domain: PSD-95, Dlg, and ZO-1. The PDZ domains are linear motifs of approximately 90 residues in size and adopt a common folding, consisting of a Beta-barrel capped by alpha-helices (Cabral et al., 1996). The PDZ proteins usually have multiple PDZ domains and additional protein-protein interaction elements, such as SH3, L27 or leucine-rich repeat (LRR) domains, allowing them to form interactions with many protein partners simultaneously. They are typically cytoplasmic and membrane adapter proteins that are involved in a variety of different cellular processes, such as regulation of cell-cell junctions, cell polarity and signal transduction pathways. In humans, over 900 PDZ domains are found in over 400 different proteins (Lee & Zheng, 2010a; Spaller, 2006).

The predominant function of PDZ domains is to bring signaling pathway components into proximity. The ligand is arranged in an antiparallel way with respect to the PDZ domain strand (Doyle et al., 1996) and is referred to as a PDZ domain-binding motif (PBM) (Figure 9). They are usually located at the extreme carboxyl terminus of a target protein, but in some cases the peptide sequence can have an internal location. The carboxyl-terminal PBMs have been grouped into three general specificity classes: type I PBM (-X-S/T-X-Φ-COOH), type II PBM (-X-Φ-X-Φ-COOH), and type III PBM (-X-D/E-X-Φ-COOH), where X is any residue and Φ is a hydrophobic residue (Hung & Sheng, 2002; Nourry et al., 2003; Tonikian et al., 2008) (Figure 9). From the structural point of view, the PBM sits in the hydrophobic groove of the PDZ domain formed between one of the β sheets (βB) and the α helix (αB), with a highly conserved carboxylate

binding loop at the base. Even though the consensus PBM is formed by the last four residues, it has been shown that PDZ proteins can recognize up to the last nine residues at the carboxyl termini of proteins and that 16 distinct specificity classes can be identified based on this (Lee & Zheng, 2010a; Tonikian et al., 2008; Töpffer et al., 2007). It has been reported that the amino acids upstream of the PBM may contribute to the specificity of PDZ substrate binding and that additional phosphorylation of the core PBM or upstream residues can have a negative or positive regulatory effect on ligand binding (Fuh et al., 2000; Laura et al., 2002; Lee & Zheng, 2010a; Schultz et al., 1998; Songyang et al., 1997).

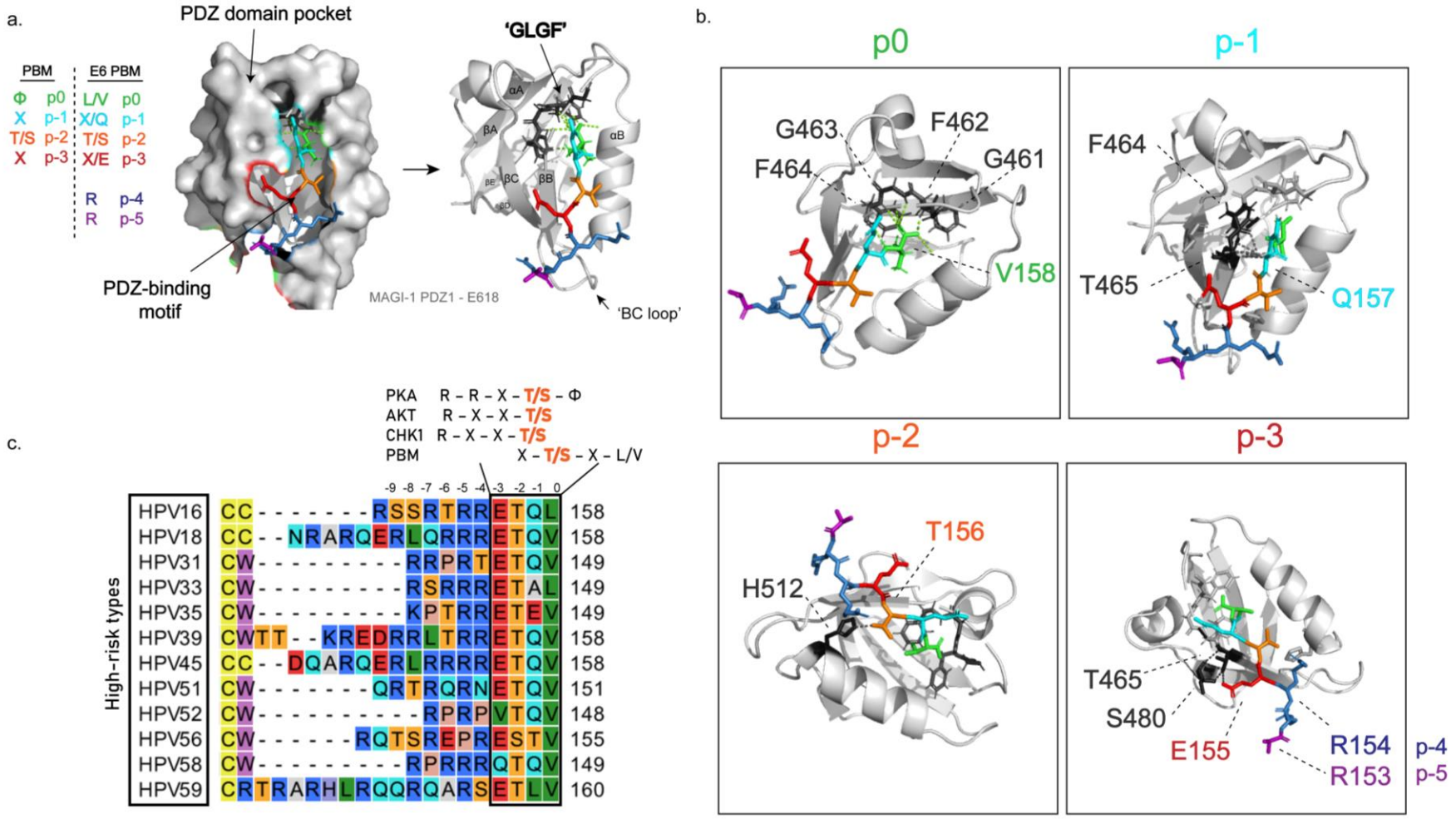

a.
PDZ domain pocket
'GLGF'
PBM
E6 PBM
Φ p0
X p-1
T/S p-2
X p-3
L/V p0
X/Q p-1
T/S p-2
X/E p-3
R p-4
R p-5
αA
βA
αB
βE
βC
βB
αC
PDZ-binding motif
MAGI-1 PDZ1 - E618
'BC loop'
b.
p0
p-1
G463
F462
F464
G461
V158
F464
T465
Q157
p-2
p-3
T156
H512
T465
S480
E155
R154
R153
p-4
p-5
c.
PKA R – R – X – T/S – Φ
AKT R – X – X – T/S
CHK1 R – X – X – T/S
PBM X – T/S – X – L/V
-9 -8 -7 -6 -5 -4 -3 -2 -1 0
High-risk types
HPV16 CC-------RSSRTRRETQL 158
HPV18 CC--NRARQERLQRRRETQV 158
HPV31 CW---------RRPRTETQV 149
HPV33 CW---------RSRRRETAL 149
HPV35 CW---------KPTRRETEV 149
HPV39 CWTT--KREDRRLTRRRETQV 158
HPV45 CC--DQARQERLRRRRETQV 158
HPV51 CW-------QRTRQRNETQV 151
HPV52 CW---------RPRPVTQV 148
HPV56 CW------RQTSREPRESTV 155
HPV58 CW---------RPRRRQTQV 149
HPV59 CRTRARHLRQQRQARSETLV 160

Figure 9.- Structure and sequence determinants of the E6 PBM and the interaction with PDZ domains. (a) The PDZ-binding motif is a class I PBM, which has four well conserved residues which fit into the hydrophobic pocket of the PDZ domain. The PDZ domain itself is an evolutionarily conserved domain of approximately 90 amino acids folded into six β-sheets (βA–βF) and two α-helices (αA–αB), named for its presence in PSD95, DLG, and ZO-1 proteins. The PBM sits into the hydrophobic groove of the PDZ domain formed between one of the β sheets (βB) and the α helix (αB,) and it is arranged in an antiparallel fashion with respect to the PDZ domain strand, in a so-called -augmentation process (Mamonova et al., 2017). The carboxyl group of the PBM is well anchored into the GLGF motif on the PDZ, forming a hydrogen bond with the amide nitrogen group in the GLGF motif located prior to the βB strand. (b) Each position of the E6 PBM is shown. The position p-0 (green) L/V mutation is important for Scribble/16E6 and Dlg1/18E6 specific binding, since it directly contribute to the GLGF pocket (black). The position p-1 have been shown to contribute to the specificity of E6 for targeting a particular PDZ domain (difference between PDZ2 and PDZ 3 of DLG). The residue in p-2 forms a hydrogen bond with the highly conserved histidine residue (H512) on the αB chain of the PDZ. Because of this, phosphorylation of threonine residue results in a steric hindrance with the peptide binding groove of the PDZ domain. The last residue of the core PBM on p-3 forms hydrogen bonds with all 3 PDZ domains (MAGI and DLG). In the case of PDZ1-MAGI-1, it binds important residues (T465/S480) from βB and βC. In the PBM canonical sequence, the E6-glutamine residue is normally an X, but in E6, this residue is present in all HR types, implying that it contributes to specific binding (Charbonnier et al., 2011; Liu et al., 2007; Zhang et al., 2007). (c) Amino acids outside the E6 PBM also contribute to PDZ substrate specificity of binding, and phosphorylation of the PBM can act as a negative or positive regulator of PDZ binding. Indeed, the phosphorylation in position p-2, generates a conformational switch that decouples the dynamics in the βC-αA region affecting the ligand-binding site. In the case of HPV, not only variation in the four last residues determine substrate specific, but residues upstream on positions p-4, p-5, and p-6, have been determinant to confer a higher binding promiscuity to high-risk types.

The PDZ-PBM interactions have evolved in biological systems via rewiring of interactions. One third of the human PDZ ligands obtained their PBMs via carboxyl-terminal sequence mutations, providing evolutionary advantages to the PDZ domain-mediated interactions (Kim et al., 2012). Indeed, the linear nature of the PBM is an efficient mechanism to increase the number of interactions; since they rarely disrupt the protein structure and a new interaction can be accomplished by few amino acid changes (Neduva et al., 2005; Neduva & Russell, 2005). Additionally, short linear motifs that are intrinsically unstructured can acquire a specific structure when bound to PDZ proteins (Münz et al., 2012). As a consequence of this, PDZ domains have diversified their binding partners in the organization of various signaling complexes, from receptors to downstream signaling relays. Moreover, the ligands of PDZ proteins show tissue-specific expression patterns that allow the formation of tissue-specific cell signaling complexes.

The role of PDZ proteins in cell polarity regulation and tumorigenesis

The control of cell polarity involves the asymmetric distribution of macromolecules to specific membrane domains. It is essential for normal cellular function and morphogenesis during development in multicellular organisms. There are four types of cell polarity: apico-basal cell polarity, involved in epithelial polarity; planar cell polarity, which is the polarity across the plane of the epithelium; asymmetric cell division, which is involved in the self-renewal of stem cells and differentiation; and front-rear cell polarity, involved in directed cell migration (Gandalovičová et al., 2016). All of these are defined by the type of cells and the mechanism that they regulate. The key to the establishment and maintenance of cell polarity in apico-basal, front-rear and asymmetric cell division polarity types, are the antagonistic interaction between two polarity modules: the Scribble module, containing the Scrib, Dlg and Lgl proteins (Elsum et al., 2012; Humbert et al., 2008; Humbert et al., 2015; Stephens et al., 2018); and the Par module comprising the Par3 and Par6 proteins, the PKCα kinase and the small GTPase Cdc42 (Chen & Zhang, 2013). The Scribble module proteins are localized to the basolateral cortex in epithelial cells. The antagonistic interaction between the Scribble and Par modules is mediated by PKCα binding to, and phosphorylation of, Lgl, excluding it from the plasma membrane. Conversely, Lgl binds to PKCα and inhibits its activity by binding to Par6 in the PKCα-Par6 complex, thus

competing with the binding of Par3 and preventing the membrane accessibility of the PKCα-Par6 complex. At the same time, Par3 binding to the PKCα-Par6 complex allows the PKCα-phosphorylation of Numb (Wirtz-Peitz et al., 2008).

Additionally, in apico-basal cell polarity in epithelial cells, the Par module interacts with another cell polarity module, the Crumbs module, consisting of the Crb, Pals and Patj proteins. The Scribble, Par and Crb modules also regulate cellular signaling pathways to control cell proliferation, survival and migration and, consistent with these roles, these cell polarity proteins are frequently deregulated in cancer (Elsum et al., 2012; Martin-Belmonte & Perez-Moreno, 2012). In human cancer, cell polarity gene function is perturbed by mutations and alteration in protein localization and protein degradation (Banks et al., 2012; Ganti et al., 2015; Tervonen et al., 2011). In fact, the HPV oncoproteins mediate the degradation of both Dlg and hScrib PDZ proteins resulting in more aggressive cancers, since the loss of function of Scrib, Dlg or Lgl results in excessive cell proliferation and the formation of neoplastic tumours with aberrant differentiating and cell morphology. There are four mammalian orthologues of Dlg (Dlg1, Dlg2, Dlg3, and Dlg4), two of Lgl (Llgl1 and Llgl2) and only one of hScrib, that have been described as tumour suppressors, inhibiting cell proliferation and the epithelial-mesenchymal transition (EMT) (Elsum et al., 2013; Humbert et al., 2008).

The hScrib, Dlg1 and Lgl proteins do not have enzymatic activity, but they contain specific protein-protein interaction domains, that allow them to bind signaling proteins, such as kinases and phosphatases, having functions which are dependent on their cellular sublocalization. HScrib is a large multidomain scaffold protein and belongs to the LAP (LRR and PDZ) protein family. It contains 16 LRRs and 4 PDZ domains and it localizes to the basolateral membrane of epithelial cells. Dlg1 is a member of the MAGUK scaffolding protein family and has three PDZ domains, a SH3 domain, a Hook domain and a GUK domain. Lgl is not a PDZ protein but is a large multidomain protein comprising an amino-terminal region with WD40 repeats and a carboxyl-terminus harboring phosphorylation sites (Stephens et al., 2018).

In addition to their roles in cell polarity, the Scribble module proteins interact with many proteins involved in different cellular processes, such as cell adhesion, membrane trafficking, cell migration and cellular signaling. The Scrib amino-terminal LRR domain interacts with several proteins such as Llgl2, Sgt1/Hsp90, PHLPP1, the BMP receptors and the transcription factor Mad in mammalian cells, and the early endosomal protein Rab5 in Drosophila. Additionally, its PDZ domains preferentially interact with several

cell junctional proteins, the most important being β-catenin (Sun et al., 2009), the tight junction protein ZO-2 (Métais et al., 2005), and the intermediate filament protein Vimentin (Phua et al., 2009). It also associates with proteins involved in the regulation of cell migration such as β-PIX and MCC. In the case of Dlg, its PDZ domains interact with many proteins, such as *Drosophila* Vangl, which regulates planar cell polarity; human APC, which is involved in cell adhesion, and many viral proteins involved in carcinogenesis and pathogenesis (James & Roberts, 2016a). Although there is a wide range of interactors, each PDZ domain within Scrib and Dlg have shown specificity and preference for different ligands, which helps in maintaining unique binding profiles. Besides cellular interactors, many viral proteins have also been shown to target PDZ proteins, resulting in increased viral replication, infection or transformation.

PDZ proteins in virus pathogenesis and carcinogenesis

A select set of PDZ proteins are commonly targeted during infections by different viruses with quite distinct replication cycles. Around 1997, the PBMs were initially discovered in viral oncoproteins, specifically in human adenovirus (Ad) E4-ORF1, human T-lymphotropic virus type 1 (HTLV-1) Tax, and human papillomavirus E6 (Kiyono et al., 1997; Lee et al., 1997; Rousset et al., 1998). Later, PBMs were found in proteins from non-transforming viruses such as influenza A virus and tick-borne encephalitis virus (TBEV), which target many of the same PDZ proteins as do the PBMs from viral oncoproteins. The association of some viral PBMs with PDZ proteins results in their loss of function, either through degradation or sequestration in cellular compartments, and in other cases, results in the gain of function of the cellular protein. Many studies have shown that viral proteins from eight virus families target cellular PDZ domain-containing proteins (James & Roberts, 2016a; Javier & Rice, 2011).

The first virus-encoded PBM (ATLV) was found on the extreme carboxyl terminus of E4-ORF1 protein from Adenovirus type 9 (Ad9), a human virus commonly associated with benign eye infections, which causes mammary tumours in experimentally infected female rats (Javier, 2008). Its oncogenic potential is determined by the PBM of the E4-ORF1 protein, which interacts with Dlg1 (Lee et al., 1997) and also mediates the binding to MUPP1, PATJ, MAGI-1 and ZO-2 (Glaunsinger et al., 2000, 2001; Latorre et al., 2005; Lee et al., 2000). In epithelial cells, the E4-ORF1 protein also disrupts the TJ by a PBM-dependent mechanism (Latorre et al., 2005), and causes the loss of apico-basal polarity, preventing the establishment of anterior-posterior cell polarity.

Furthermore, the Ad9 E4-ORF1 promotes constitutive growth factor-independent PI3K activation at the plasma membrane through a PBM-dependent mechanism that is crucial for its oncogenic potential and triggers the activation of downstream targets, including AKT/PKB (Frese et al., 2003; Kong et al., 2014; Kumar et al., 2014).

The Human T-cell Leukemia Virus (HTLV-1) is an important member of the retroviridae family that replicates in CD4+ T cells and causes highly aggressive adult T cell leukemia. It encodes the envelope (Env) protein that also contains a carboxyl-terminus class I PBM, which contributes to the activity of Env to trigger cell-to-cell fusion; and a PDZ-binding protein Tax, which is a transcriptional activator and has a class I carboxyl-terminal PBM (ETEV) (Lee et al., 1997; Rousset et al., 1998). The Tax PBM seems to play an important role in its ability to transform cells and in driving T cell proliferation. Dlg1 and hScrib have been identified as strong binding partners of the Tax PBM and they are both inactivated by sequestration within detergent-insoluble complexes in cells (Hirata et al., 2004), affecting the formation of signaling complexes at specific sites in the cell. Furthermore, experiments using the yeast two-hybrid system were used to identify six other Tax-interacting PDZ proteins: Dlg4, β1-syntrophin, Lin-7, TIP-1, TIP-2/GIPC, and TIP-40 (Rousset et al., 1998).

Like the HPV E6 protein, the Tax PBM is also susceptible to regulation by phosphorylation. Casein kinase 2 phosphorylates the threonine residue within the Tax PBM and negatively regulates its binding to PDZ proteins, suggesting that timing of PDZ targeting may be important in the HTLV-1 replication cycle and host transformation (Bidoia et al., 2010).

Another indirect cancer-causing virus is the retrovirus HIV-1, which infects CD4 positive T cells and causes AIDS. A PBM-independent interaction between Dlg1 and the HIV-1 Gag protein reduces HIV-1 virion infectivity (Guth & Sodroski, 2014; Henning et al., 2010). The HIV-1 viral envelope glycoprotein (Env) also recruits the PDZ protein syntenin-1 to the plasma membrane during HIV-1 attachment and associates with the main HIV-1 receptor CD4. Moreover, in polarized epithelial cell cultures, HIV-1 infection causes displacement of the PDZ protein ZO-1 from the tight junctions in the apical region of the membrane, followed by a marked reduction in the transcription of ZO-1 and other tight junction proteins (Gordón-Alonso et al., 2012; Henning et al., 2011; Perugi et al., 2009; Valenzuela-Fernandez et al., 2005)

The targeting of PDZ proteins is a common function amongst viruses classified as human carcinogens by the World Health Organization (Bouvard et al., 2009). The

Hepatitis B virus (HBV) from the Hepadnaviridae family causes acute and chronic hepatitis and is a major risk factor for development of cirrhosis or hepatocellular carcinomas. The HBV core protein interacts with the PDZ protein GIPC1 (TIP-2) (Razanskas & Sasnauskas, 2010) and this interaction is mediated by a variant class III carboxyl-terminal PBM (SQAR) in the viral core protein and the single PDZ domain in GIPC1. This interaction is interesting since the PBM of high-risk HPV E6 and HTLV-1 Tax likewise mediate binding to cellular GIPC1 (Favre-Bonvin et al., 2005; Rousset et al., 1998). Given that the GIPC1 adaptor protein tethers proteins to myosin VI and participates in vesicle recycling of membrane receptors, one possible function for the HBV core-GIPC1 complex may be to facilitate transport of HBV capsids from the cytoplasm into the nucleus.

The Hepatitis C virus (HCV) is also a common risk factor for hepatocellular carcinoma. The virus primarily infects the polarized epithelium in the liver, and disruption of tight junctions is necessary for entry and cell-to-cell transmission. Expression of the HCV core protein disrupts the apicobasal polarity of the epithelia, mislocalizes hScrib and decreases expression of Dlg1 protein at the cell-cell contact area (Awad et al., 2013).

The PDZ-PBM interaction has also been shown to be important for the replication, transmission and pathogenesis of viruses that do not cause cancer. In the case of the neurotropic Rabies virus from the Rhabdoviridae family, the viral envelope G protein has a carboxyl-terminus class I PBM (QTRL) which interacts with the PDZ domain of the serine-threonine kinase MAST2, leading to the disruption of the MAST2-PTEN complex that is involved in antiapoptotic AKT activation and neuronal survival, promoting neuronal death. Furthermore, in the attenuated strain, a mutation within the G protein PBM (ETRL) confers an expansion of the PDZ proteins bound, including Dgl2, MUPP1, and the tyrosine phosphatase PTPN4 (Préhaud et al., 2010; Terrien et al., 2012).

The type A influenza viruses from the Orthomyxoviridae family also contain a carboxyl-terminus canonical class I PBM at the NS1 protein, which represents a target for attenuation of pathogenicity. In fact, the PBM of NS1 protein has been shown to be different between avian and human viruses (Obenauar et al., 2006). The avian PBM ESEV specifically mediates the *in vitro* association with at least five cellular PDZ proteins: Scrib, Dlg1, MAGI-1, MAGI-2, and MAGI-3 (Liu et al., 2010; Thomas et al., 2011). The NS1 ESEV PBM partly blocks the proapoptotic function of hScrib during viral infection, probably through its relocalization from the plasma membrane into

cytoplasmic puncta, protecting virus-infected cells from apoptosis. It has also been reported that removal of the PBM decreases the efficacy of influenza H1N1 virus transmission and replication. Indeed, when the H1N1 PBM is switched for the sequence of an avian-specific strain, virus virulence is increased (Jackson et al., 2008; Kim et al., 2014; Soubies et al., 2010). In the case of the highly pathogenic strain H5N1, the NS1 PBM interacts with the PDZ domains of several PDZ proteins (Golebiewski et al., 2011; Thomas et al., 2011).

Additionally, members of the Flaviviridae family also contain internal PBMs within the methyltransferase domain of Tick-borne encephalitis virus (TBEV) NS5 protein through which it binds the fourth PDZ domain of hScrib (Werme et al., 2008), directing NS5 to the plasma membrane and inhibiting interferon-mediated JAK-STAT signaling. In vitro binding assays have also shown that the TBEV NS5 utilizes its internal PBM to bind to the cellular TJ-associated PDZ protein ZO-1 in the plasma membrane and the neuron-specific RIMS2 PDZ protein. Furthermore, the Dengue virus (DV) NS5 protein, like TBEV NS5, uses an internal PBM to bind to ZO-1, but rather associates with it in the nucleus (Melik et al., 2012).

The SARS coronavirus from the Coronaviridae family causes severe acute respiratory infections and its pathogenicity depends on the carboxyl-terminal class II PBM (DLLV) of its envelope (E) protein that may mediate binding to the single PDZ domain of cellular PALS1 (Teoh et al., 2010), a key component of the Crumbs polarity complex that controls tight junction formation and apical-basal polarity establishment in epithelial cells. Redistribution of PALS1 to the endoplasmic reticulum-Golgi intermediate compartment and the Golgi region, delayed TJ formation and polarity establishment in SARS-infected MDCK epithelial cells. Recombinant SARS-CoV expressing the envelope E protein lacking the PBM had no effect on viral growth in a murine infection model. The E protein can also induce pro-inflammatory cytokine expression, possibly through PBM binding of the PDZ protein syntenin-1. Both PBM functions could be determinants of the lung pathobiology associated with SARS-CoV infection (Jimenez-Guardeño et al., 2014; Nieto-Torres et al., 2014; Teoh et al., 2010).

As mentioned above, the PBMs from diverse families of pathogenic viruses target many of the same cellular PDZ proteins. The internal location of the PBM on some viral proteins or the PBM swapping from E6 to E7 protein (e.g. RhPV-1) illustrate the evolutionary pressure on viruses to bind and perturb the function of key cellular PDZ proteins. This observation parallels a well-known common theme in virology, in which

many otherwise unrelated proteins from DNA and RNA tumour viruses share common abilities to target the cellular pRb and p53 tumour suppressors, to dysregulate the cell cycle and inhibit apoptosis, which are important for viral replication. Likewise, the many viral PBMs that target common PDZ proteins, such as Dlg1, hScrib, and the MAGI proteins suggest that the cellular processes regulated by these PDZ proteins, especially cellular polarity and apoptosis, are of fundamental importance for viral replication and virus-host interactions.

PDZ targeting in Papillomavirus-mediated carcinogenesis

A functional class I PBM located at the carboxyl terminus of high-risk, but not low-risk, HPV E6 proteins was first reported in 1997 by Lee et al. and shortly thereafter by Kiyono et al. (Kiyono et al., 1997; Lee et al., 1997). Although this PBM sequence is conserved in all high-risk types, and binding and degradation of PDZ proteins represents an oncogenic signature; it has been suggested that the ability to degrade PDZ proteins was acquired prior to the oncogenic trait by the ancestors of both high-risk and low-risk types. Evidence of this is in the presence of an ancestral or non-canonical PBM in the non-cancer-causing HPV-40 (Figure 2), which can bind and degrade MAGI-1 (Van Doorslaer et al., 2015). This, and the common targeting of PDZ proteins by different oncogenic and non-oncogenic viruses, suggest that this trait allowed these viruses to colonize a new cellular niche, such as the transformation zone; but for viral fitness and survival they adapted to this site by acquiring additional functions associated with cell transformation, such as hTERT activation and p53 degradation (Burk et al., 2009; Van Doorslaer et al., 2015).

The PBM function is critical for the vegetative phase of the HPV life cycle. Many studies based on human keratinocytes transfected with the complete HPV genome, but with the E6 PBM deleted, have shown that the mutant genome is unable to support viral genome amplification, expression of the viral late proteins or episomal maintenance (Delury et al., 2013b; Lee & Laimins, 2004; Nicolaides et al., 2011). The various life cycle defects are correlated with a marked reduction in proliferation of the mutant genome-containing cells, indicating that the E6 PBM regulates proliferation of the viral genome-containing cells and that this is important for multiple phases of HPV DNA replication. Interestingly, although E6 mediates the degradation of the tumour suppressor p53 in infected cells, further depletion of p53 protein by using p53-targeting siRNAs, or by introduction of a dominant negative p53, into cells harboring E6-PBM mutant HPV-16 genome-containing cells, rescues maintenance of the viral genomes,

suggesting that there is mutual cooperation between these two E6 functions in viral genome maintenance during the virus life cycle (Brimer & Vande Pol, 2014).

In agreement with the life cycle studies, expression of E6 in mammalian keratinocytes showed that the E6 PBM promotes cell proliferation, but also the acquisition of phenotypes linked to invasive and metastatic growth of tumours (Jing et al., 2007; Nguyen et al., 2003; Spanos et al., 2008; Watson et al., 2003). Additionally, the motif is necessary for the morphological transformation and induction of tumorigenesis of rodent cell lines and contributes to tumour development in a transgenic mouse model of cervical carcinogenesis (Kiyono et al., 1997; Shai et al., 2007). However, the motif is not required for the immortalization of keratinocytes, suggesting that E6 PBM function is of more importance in the later stages of HPV-driven carcinogenesis (Muench et al., 2009). The link between this HPV function and cell transformation is supported by the discovery that the E7 protein of the Rhesus papillomavirus type 1 virus (RhPV1/MmPV1) drives mucosal neoplasia and cervical cancer in its host, *Rhesus macaca* (Tomaić et al., 2009). The RhPV-1 E6 protein has no PDZ-binding motif, but instead there is evolutionary conservation of the PDZ-binding activity due to a PDZ-recognition site (A-S-R-V) on the carboxy-terminal of its E7 protein, that allows its binding to the Par3 protein, which controls the same polarity regulation pathway as Dlg1 and hScrib, and thus confers transforming capacity. Moreover, RhPV-1 targets Par3 for proteosome-mediated degradation in a PDZ-dependent manner, but the mechanism by which it degrades Par3 is unknown. This shows the critical role of the Crumbs/Par/Scribble complex in the evolutionary conservation of the PBM (Tomaić et al., 2009).

To date, nineteen PDZ proteins recognized by different E6 proteins have been identified and, in most cases, binding leads to degradation via the proteasome (Kuballa et al., 2007; Massimi et al., 2008; Thomas et al., 2016) (Figure 10). The targeted PDZ proteins are largely involved in cell polarity, cell-cell attachment and organization of cell signaling pathways. Notably, the components of the three apico-basal polarity complexes, Crumbs, Par and Scribble are recognized by E6, suggesting that deregulation of pathways controlled by these complexes has an important function in transformation.

The Scribble component Dlg1 was the first PDZ protein identified as being an HPV E6 binding partner and targeted for proteasomal degradation (Gardiol et al., 1999; Kiyono et al., 1997). During epithelial differentiation, the localization of Dlg1 changes to

cytoplasmic and nuclear. The nuclear phospho-Dlg1 forms are preferentially targeted for degradation by HPV16 E6 (Massimi et al., 2004; Narayan et al., 2009; Roberts et al., 2007), suggesting that nuclear Dlg1 may have tumour suppressor activity (Roberts et al., 2007). Additionally, E6/Dlg1 functional complexes have been identified in HPV16-positive cervical cancer cells, possibly upregulating RhoG activity by the association of the E6/Dlg1 complex with the RhoG guanine exchange factor (SGEF). This E6/Dlg1/SGEF complex contributes to the invasive phenotype of the cells, indicating that Dlg1 acquires oncogenic functions in the presence of E6 (Subbaiah et al., 2012). Moreover, in HPV-positive cervical cancers, the gap junctions are lost and E6 has been found to be in complex with Dlg1 and Cx43 (gap junction component connexin protein) in these cells, possibly inhibiting normal trafficking of connexin to the cell membrane (Macdonald et al., 2012; Sun et al., 2015). Gap junctions play an important role in cell invasion, and the E6-Dlg1-Cx43 complex may contribute to metastatic development in cervical cancer cells. Therefore, in HPV infections the E6 PBM targets the tumour suppressor forms of Dlg1 involved in the negative regulation of cell proliferation, but later, during disease progression, Dlg1 acquires oncogenic functions mediated by interaction with E6, via mislocalization or stabilization of specific pools of Dlg1 (Watson, 2002).The other Scribble component that E6 recognizes is hScrib and this interaction disrupts hScrib localization at the periphery of epithelial cells and induces loss of tight junction integrity (Nakagawa & Huibregtse, 2000). Furthermore, it has been highlighted the importance of the correct level of protein expression and localization of hScrib in pro-oncogenic signaling through the mTORC1 pathway. Indeed, it was found that hScrib can directly affect the levels of E6 mRNA expression, and by maintaining active mTORC1 signaling can also contribute towards enhancing the rates of E6 protein translation. Evidence of this, is that ablation of hScrib has a significant negative effect on the levels of HPV-18 E6 protein, indicating a role in maintaining high levels of E6 protein expression (Kranjec et al., 2016). E6 also targets several other PDZ proteins associated with tight junctions, including MAGI-1, MUPP1, PAR3 and PATJ (Facciuto et al., 2014; Lee et al., 2000; Storrs & Silverstein, 2007). Recent findings have shown that introduction of a mutant form of MAGI-1, unable to be targeted by E6 for degradation, enhances the ability of HPV-18 positive cervical cancer cells to form complexes at the tight junction (Kranjec et al., 2014). These studies also identified roles for MAGI-1 in the negative control of cell proliferation and as a regulator of apoptosis, indicating that E6 may target different

functional pools of MAGI-1 relevant at different stages of the virus life cycle and disease progression (Kranjec et al., 2014).

Protection of HPV-infected cells against apoptosis also involves the activation of the PI3K-AKT and NF-κB signaling pathways in a PDZ-dependent manner. E6 targets the PDZ protein Na+/H+ exchange regulatory factor (NHERF1) to attenuate PI3K signaling through the negative regulator PTEN. HPV E6 is known to stimulate the key regulator of cellular metabolism mTORC1 leading to activation of cap-dependent translation, and the E6 PBM function has been shown to contribute to the enhancement of translation (Accardi et al., 2011; James et al., 2006; Spangle et al., 2012). Furthermore, the structural-related protein to NHERF-1, NHERF2, have been recently shown to be targeted by HPV E6 in a PBM-dependent manner and to be degraded via the proteasomal pathway. The PDZ-containing protein NHERF-2 can behave as a tumour suppressor. It negatively regulates endothelial proliferation by the upregulation the expression of p27 protein. E6-mediated degradation of NHERF-2 has been proved to indirectly lead to the downregulation of p27, increasing the expression of cyclin CDK1 and CDK4 proteins, thereby, promoting cellular proliferation. Indeed, this complements the previously known mechanism of HPV E7 to inactivate p27 by preserving it in the cytoplasm in, which also results in increased cellular proliferation (Saidu et al., 2019; Yan et al., 2010).

Even though many targets have been identified, it is still not known which ones are more relevant for carcinogenicity. In metastatic cervical cancers, the reduction in expression of the polarity proteins Dlg1 and hScrib could be E6-mediated via proteasomal degradation (Cavatorta et al., 2004; Nakagawa et al., 2004). Indeed, levels of both proteins are rescued upon E6 silencing in HPV-positive cervical cancer cell lines, although the effect is more marked for MAGI-1, since membrane-bound and nuclear pools of the protein are restored and the protein is re-localized to the tight junctions (Kranjec & Banks, 2011). In contrast, the level of expression and localization of endogenous Dlg1 and hScrib proteins is not altered in normal human keratinocytes expressing E6 alone or E6 and E7 together, and there are no changes in the expression or localization of these proteins when the E6 PBM is deleted (Choi et al., 2014; Simonson et al., 2005). Therefore, it is possible that there is a differential regulation of PDZ substrates by E6 at different stages of HPV pathogenesis.

The mechanism of HPV targeting of PDZ substrates is even more complex since the E6* spliced form of E6 lacks the PBM and still can target Dlg1, PATJ, MAGI-1 and, to

a lesser extent, hScrib for proteasomal degradation (Pim et al., 2009; Storrs & Silverstein, 2007). Moreover, there is a cooperative regulation since it has been reported that HPV-16 E6 and E7 cooperate to induce the degradation of NHERF1. E7 promotes the accumulation of phosphorylated forms of NHERF1 thorough activation of cyclin-dependent kinases and these modified forms are preferentially degraded by the E6 PBM (Accardi et al., 2011).

Furthermore, the complexity of the PBM in terms of the sequence variation among the different high-risk types influences the substrate specificity. Indeed, there is a correlation between the functional flexibility of E6 and the risk-classification, since carcinogenic types, specifically from the group 1, interact with a greater number of PDZ targets. (Accardi et al., 2011; Handa et al., 2007; Kranjec & Banks, 2011; Thomas et al., 2016). A reported example is the variable preferences for binding PDZ proteins of HPV-16 and HPV-18. HPV-16 E6 preferentially binds hScrib over Dlg1 and HPV-18 binds Dlg1 over hScrib, and this preference is mainly due to the last residue (Leucine/Valine) of the PBM, notably affecting the specificity for Dlg1 or hScrib (Thomas et al., 2005). Whether this is relevant to the carcinogenic potential of the virus is not clear, but it is clear that the sequence variability, splicing and cooperative action of E6 and E7 are mechanisms that can control PDZ protein recognition.

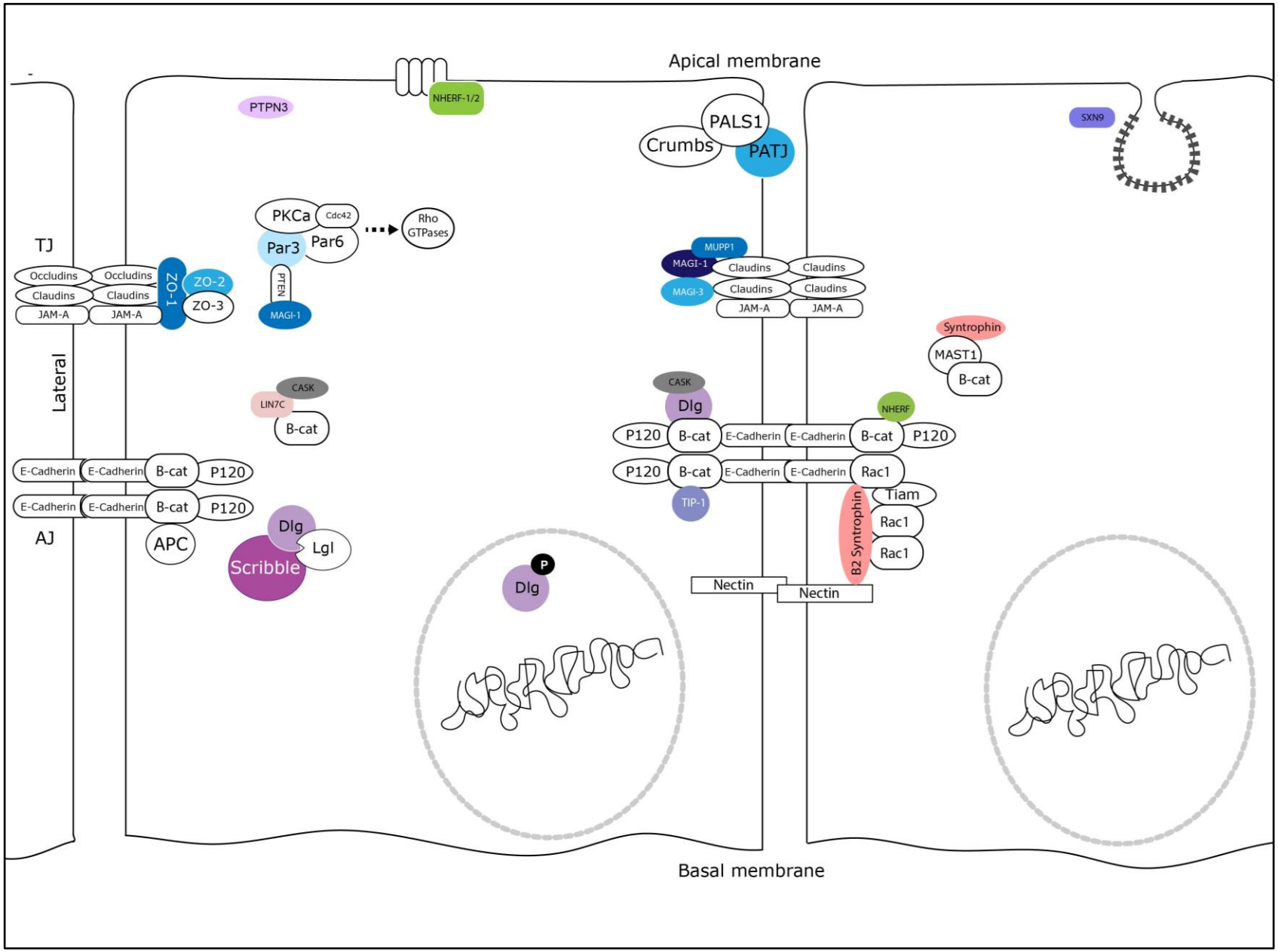

Apical membrane
PTPN3
NHERF-1/2
PALS1
Crumbs
PATJ
SXN9
PKCa
Cdc42
Rho GTPases
Par3
Par6
PTEN
MAGI-1
TJ
Occludins
Occludins
Claudins
Claudins
JAM-A
JAM-A
ZO-1
ZO-2
ZO-3
MUPP1
MAGI-1
MAGI-3
Claudins
Claudins
Claudins
Claudins
JAM-A
JAM-A
Syntrophin
MAST1
B-cat
Lateral
CASK
LIN7C
B-cat
CASK
Dlg
NHERF
P120
B-cat
E-Cadherin
E-Cadherin
B-cat
P120
E-Cadherin
E-Cadherin
B-cat
P120
P120
B-cat
E-Cadherin
E-Cadherin
Rac1
TIP-1
Tiam
Rac1
Rac1
B2 Syntrophin
E-Cadherin
E-Cadherin
B-cat
P120
E-Cadherin
E-Cadherin
B-cat
P120
AJ
APC
Dlg
Lgl
Scribble
P
Dlg
Nectin
Nectin
Basal membrane

Figure 10.- The human papillomavirus oncoproteins target different cell polarity proteins. The figure shows various proteins comprising the three major complexes that regulate cell polarity. In the apical membrane, E6 targets the Crumbs (CRBS) complex by affecting PATJ protein. In the sub-apical membrane, E6 targets the Par complex (Par 3) and in basolateral membrane the Scribble complex (hScrib and Dlg1). These complexes interact through a series of mutually antagonistic interactions. The HPV E6 and RhPV E7 proteins target diverse components of this cell polarity control network for proteasomal degradation, perturbing their levels of expression or localization. The protein targeted by E6 and E7 are shown in colors.

Essential roles of p53 protein

TP53 has been mainly studied as a tumour suppressor gene that detects oncogenic events in cancer cells, induce them to senescence and them eliminate them by apoptosis. For this, it has earned the name of 'Guardian of the genome' (Lane, 1992). In many types of cancer, the p53 activity is compromised, either by mutations in the TP53 gene or by changes in its modulators. Besides the tumour suppressive activity of p53, it has also been involved with processes like DNA damage response, metabolism, aging, stem cell proliferation and fertility.

The p53 protein has tumour-suppressor activity, working mainly as a highly regulated transcription factor with transactivation and trans repression activities (Beckerman & Prives, 2010; Lane & Levine, 2010; Riley et al., 2008; Vousden & Prives, 2009). It can regulate the expression of several genes involved in regulating cell growth, division, survival and programmed death. Under normal conditions, p53 is a short-lived protein that is regulated mainly through changes in its protein stability (Hu et al., 2012). However, in response to stress stimuli, for example DNA damage, p53 is induced, its degradation is blocked and its cellular levels increase, which can block uncontrolled proliferation by inducing growth arrest, senescence or apoptosis (Meek, 2015). The RING-finger type E3 ubiquitin ligase, Mdm2 (murine double minute 2) is the main regulator of p53. The cellular low levels of p53 are maintained by the Mdm2, which at high levels, polyubiquitinates p53 at six carboxy-terminal lysine residues and targets it for proteasomal degradation (Brooks & Gu, 2006; Lee et al., 2009; Meek & Anderson, 2009; Rodriguez et al., 2000). However, lower levels of Mdm2, also

monoubiquitinates p53 facilitating its nuclear export (Li et al., 2003). Indeed, Mdm2 and p53 act within a negative-feedback loop in which p53 transactivates MDM2 expression through the stronger of two promoters in the MDM2 gene, regulating the levels of its negative regulator, and restoring its homeostatic levels after the inducing signal is gone. Therefore, the induction mechanism of p53 happens mainly through blocking the p53-MDM2 interaction. Moreover, several other p53-targeted ubiquitin ligases, including Pirh2, COP1, CHIP, ARF-BP1, E6AP, TOPORS, TRIM24 and MKRN1 also contribute to p53 turnover .

Generally, the transcriptional activity of p53 is downregulated in three ways in homeostasis. The above-mentioned, ubiquitin mediated proteasomal degradation by Mdm2; the decrease in nuclear p53 levels because of the exposure of the nuclear export sequence of p53 or Mdm2; or the transcriptional repression of chromatin-associated p53 by Mdm2-Mdmx-p53 complex formation . By contrast, under stress, degradation and nuclear export of p53 are suppressed, and the nuclear import of p53 is enhanced, resulting in its nuclear accumulation. The transcriptional levels of p53 downstream target genes can be increased by p53 nuclear accumulation (Gu & Zhu, 2012).

Although many studies have focused on the roles of p53 under various stress stimuli and the different mechanisms used to achieve p53 induction (Meek, 2015); the basal non-induced levels of p53 have additional roles in regulating a range of essential genes in different cellular processes, one of them, particularly important of HPV life cycle, proliferation and differentiation of stem cells . Moreover, p53 has an important role in the regulation of metabolism. Indeed, p53 can regulate genes involved in metabolism and mitochondrial respiration, and thereby reduce the flux through the glycolytic and pentose phosphate pathways, and stimulate mitochondrial function (Feng & Levine, 2010; Vousden & Prives, 2009; Vousden & Ryan, 2009). In this way p53 favors mitochondrial oxidative phosphorylation as the main way of ATP production and minimize the synthesis of substrates needed for growth and cell division. This works as a barrier of the ‘Warburg effect’, in which oncogenic processes promote aerobic glycolysis and the pentose phosphate pathway (Zhang et al., 2013). Additionally, p53 regulates the IGF-1/mTOR (insulin-like growth factor 1/mammalian target of rapamycin) pathway, controlling the routes by which proliferation, survival and energy metabolism are controlled (Feng & Levine, 2010).

Induction of p53 by PTM

The p53 protein goes through a post-translation modification cascade in normal homeostatic cells during cell cycle progression. Phosphorylation of Ser9, Ser15, Ser20 and Ser372 peaks during G1, whereas Ser37 and Ser392 phosphorylation peak during G2/M. Ser37 is the only site being phosphorylated during S phase and acetylation is mostly abundant at G0 .

Furthermore, it has been confirmed that Mdm2, CBP/p300 and p53 form a complex in unstressed cells, and phosphorylation of Ser15, Thr18 and Ser20 residues in the amino-terminal site, increase p53's affinity for CBP/p300 . The Ser15 is phosphorylated, and it is required for T18 phosphorylation, which is also required for S20 phosphorylation. Indeed, the amino-terminal phosphorylation of p53 can be classified into several clusters, depending on the kinases that phosphorylate the nucleating site . Additional phosphorylation patterns induce the acetylation of the carboxy-terminus and initiate a phosphorylation-acetylation cascade. For example, phosphorylation of S378 and T377 reduces acetylation of K373, 382, 320; and phosphorylation of S366 and T387 enhances carboxy-terminal acetylation (Sakaguchi et al., 1998)

Carboxy-terminal phosphorylation also mediates p53 ubiquitination by Mdm2. Indeed, p53 degradation goes through a series of processes that initiate with the carboxy-terminal phosphorylation, relayed by the inhibition of carboxy-terminal acetylation and ended up with ubiquitination. Acetylation is the hub of p53 transactivation and is contained within a network of various upstream and downstream modifications. In fact, all the lysine residues acetylated by p300/CBP are ubiquitination targets .

The PTM cascade of p53 is always accompanied by a binding partner cascade, indicating a role of the modifications to mediate the interaction between p53 and its partners . Although, there is an important PTM function in unstressed cells, it is widely known that p53 is modified more extensively under genotoxic stress. Being at the center of p53 activation acetylation and phosphorylation.

Induction of p53 by PTM during the DNA damage response

The p53 tumour suppressor was shown to be a phosphoprotein soon after its discovery as on oncoprotein 30 years ago. Many phosphorylation events on p53 are

stimulated by a variety of genotoxic and nongenotoxic agents , but there are differences in the degree of modification of different residues achieved using different stress-inducing agents (Figure 11).

The DNA damage response is the most studied mechanism which induces PTM of p53. The induction and activation of p53 in the DNA damage response pathway is generated by two protein kinases: ATM (ataxia telangiectasia mutated), and ATR (ataxia telangiectasia- and Rad3-related), which are activated in response to double and single-strand breaks respectively. The main function of these proteins is generating post-translational changes on p53 and its direct regulators, inhibiting the Mdm2-mediated degradation of p53. Indeed, ATM and ATR have been shown to phosphorylated Mdm2 in Ser395 and Ser407, respectively. These modifications affect the allosteric formation of Mdm2 dimer mediated through the RING domain, blocking the poly-ubiquitination of p53, therefore stabilizing it . Mdm4 is also down-regulate in response to DNA damage, through phosphorylation on Ser342 and Ser367 by CHK2 and Ser 403 by ATM, allowing 14-3-3ɣ binding and leading to ubiquitination and degradation of Mdm4, blocking the interaction with Mdm2 and thereby promoting p53 turnover (Chen et al., 2005; Pereg et al., 2006).

The phosphorylation of p53 in Ser15 by ATM and ATR protein kinases, has been considered to be an initiating and nucleating event in p53 activation, that promotes the sequential modification of many subsequent residues (Saito et al., 2003; Saito et al., 2002; Sakaguchi et al., 1998, 2000). Indeed, the phosphorylation of Ser15 occurs within the first 30 min of the DNA damage stimulus. Although the ATM activation can be transient, subsequent slower activation of the ATR occurs, providing the continuity of Ser15 phosphorylation that can endure for several hours after the initial stimulus. Furthermore, other kinases like DNA-activated protein kinase (DNA-PK) phosphorylates p53 in vitro on Ser15 and Ser37 . The effect in most of these cases is similar, the phosphorylation of Ser15 incudes the dissociation of MDM2 from p53, which results in p53 stabilization (Shieh et al., 1997).

Importantly, the phosphorylation of Ser15 initiates a series of sequential events, like allowing the recognition for CK1 kinase to phosphorylate Thr18. Thr18 potentially contributes to uncoupling p53 from degradation, by changing the electrostatic nature of the p53-Mdm2 interaction (Craig et al., 1999; Dumaz et al., 1999; Feng et al., 2009; Ferreon et al., 2009; Lee et al., 2010; Sakaguchi et al., 2000; Teufel et al., 2009). Moreover, Ser15 phosphorylation masks a nuclear export signal, contributing to the

retention of p53 within the nucleus . A subsequent, Ser20 phosphorylation event by CHK2 kinase, activated by ATM, contributes to uncoupling the p53-Mdm2 interaction, especially in combination with Ser15 and Thr18 (ElSawy et al., 2015; Zhang & Xiong, 2001). Initially, it was thought that the modification of these residues was the main mechanism for DNA damage-dependent induction of p53, however, the contribution of the Mdm2 Ser394 phosphorylation to this is undeniable and provide evidence that each phosphorylation event in different proteins, might happens in different sites and after specific stimuli .

Other residues in the TAD1 region of p53 also undergoes DNA-damage induced phosphorylation, including Ser6, Ser9, Ser33 and Ser37 . Ser6 and Ser9 were originally identified as targets of the protein kinase CK1 family members, CK1ɣ and CK1ε. P53 proteins with alanine residues substituted at these phosphorylation sites fail to interact with Smad2 protein. The protein kinases CK1γ and CK1ε are important for the phosphorylation of p53 in response to FGF signaling . However, modification of these residues does not appear to affect the interaction of p53 with Mdm2 (Lee et al., 2010; Teufel et al., 2009).

The PTMs of p53 not only down-regulate Mdm2 binding, but promote the association with key transcription factors, acting as switches between degradation and activation. As mentioned, the phosphorylation of residues in the amino-terminus of p53 (TAD1), stimulates the interaction with p300 and CBP transcriptional co-activator proteins, with Thr18 phosphorylation playing an important role, with multiple phosphorylation events, like additional Ser15 phosphorylation, also enhancing the interaction by 80-fold (Jenkins et al., 2012; Meek & Anderson, 2009). Therefore, under stress stimuli, p53 acetylation by CBP and p300 is also regulated by phosphorylation. Indeed, the six lysine residues acetylated by CBP and p300 in the carboxy-terminus of p53 are the same lysine residues that are ubiquitinated by Mdm2, making acetylation and ubiquitination of these residues mutually exclusive. Several other PTMs of p53 are induced during the DNA damage response, adding to phosphorylation and acetylation, SUMOylation, NEDDylation and glycosylation events. These modifications can vary depending on the DNA damage type, the intensity and the duration of the stimulus .

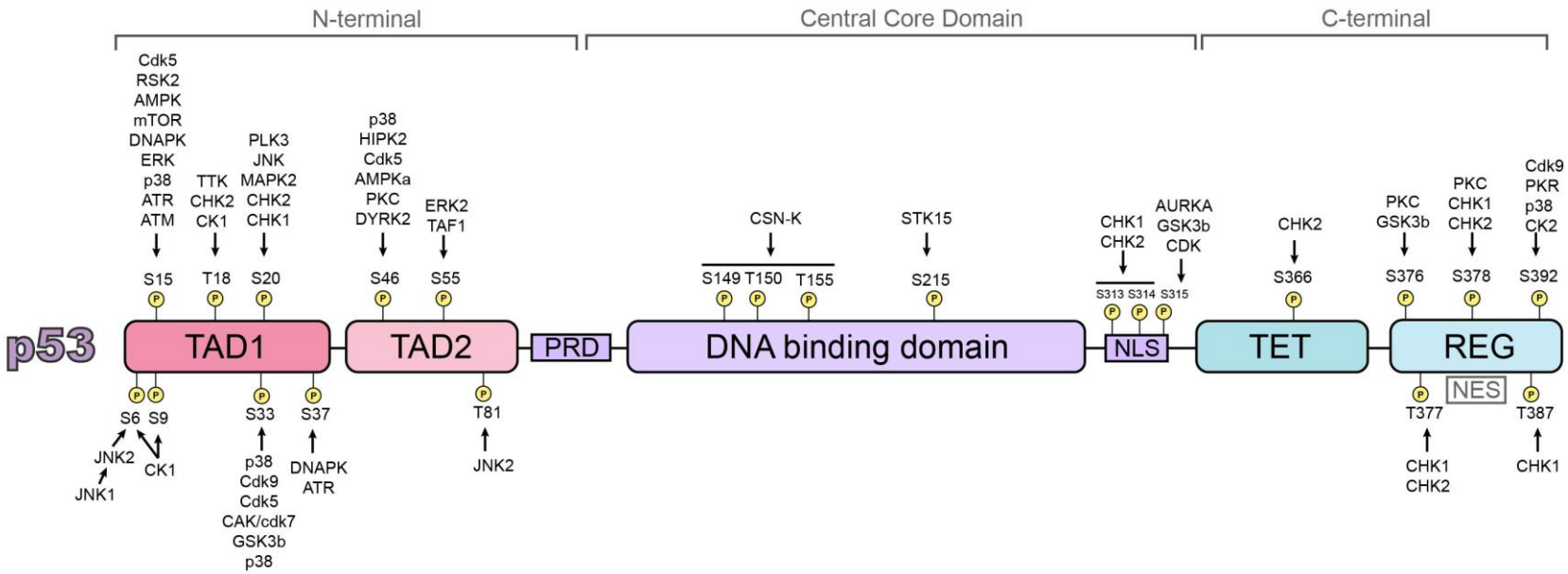

N-terminal
Central Core Domain
C-terminal
p53
TAD1
TAD2
PRD
DNA binding domain
NLS
TET
REG
NES
Cdk5
RSK2
AMPK
mTOR
DNAPK
ERK
p38
ATR
ATM
S15
TTK
CHK2
CK1
T18
PLK3
JNK
MAPK2
CHK2
CHK1
S20
p38
HIPK2
Cdk5
AMPKa
PKC
DYRK2
S46
ERK2
TAF1
S55
CSN-K
S149 T150
T155
STK15
S215
CHK1
CHK2
S313 S314
AURKA
GSK3b
CDK
S315
CHK2
S366
PKC
GSK3b
S376
PKC
CHK1
CHK2
S378
Cdk9
PKR
p38
CK2
S392
S6 S9
JNK2
JNK1
CK1
S33
p38
Cdk9
Cdk5
CAK/cdk7
GSK3b
p38
S37
DNAPK
ATR
T81
JNK2
T377
CHK1
CHK2
T387
CHK1

Figure 11.- Schematic representation of the structure of p53. Within the amino-terminal domain, there are located the transactivation domains 1 and 2 (TAD1 and TAD2 respectively), and the proline-rich domain (PRD). The DNA-binding domain comprises the central core part of p53. Furthermore, the carboxy-terminal side contains the tetramerization domain (TET), important for protein stability; and the regulatory domain (REG). The nuclear localization and export signals are shown (NLS and NES, respectively). The most common post-translation modification of p53 is phosphorylation. Following genotoxic stress and in normal homeostatic state, several kinases phosphorylate p53 at multiple serine and threonine residues leading, in the majority of cases, to p53 stabilization. Most of the sites are located in the amino-terminal region. Some protein kinases phosphorylate numerous sites and several sites are phosphorylated by more than one kinase

The fundamental role of ser15 phosphorylation

Although, most of the PTMs have been described in the context of the DNA damage response and Ser15 phosphorylation is an important event of p53 activation; recent evidence has suggested that Ser15 phosphorylation has a broader role in p53 activation. Indeed, basal levels of p53 and p53 induced by Mdm2-inhibition show detectable levels of Ser15 phosphorylation. Therefore, other mechanism also allows this to occur under different circumstances, independently of p53 low levels . Experiments with alanine substitution of Ser15, unable to be phosphorylated, impairs the growth arrest induced by p53. Conversely, the phosphomimic mutations to aspartate, maintains the p53 transcriptional and biological functions. Furthermore, the ability of p53 to be loaded in promoters like p21, ready to start transcription following a given stimulus, is impaired when Ser15 is mutated to alanine .

Ser15 is also phosphorylate through the AMPK pathway in response to glucose depletion and mediates p53-dependent metabolic arrest at G1/S, suggesting Ser15 has a critical role for cellular stresses that are independent of DNA damage (Jones et al., 2005).

Thus, the regulation of p53 activity through PTMs is part of its regulation in a non-DNA damage context. Although, the DNA damage induces p53 mainly through a controlling event at Mdm2 (Gannon et al., 2012).

AIMS OF THE STUDY

It has been previously shown that E6 phosphorylation within the PBM is crucial for the PDZ-to-14-3-3 switch, and therefore, E6's functional flexibility. Indeed, this is also reflected on its functionality and oncogenic properties.

This thesis presents the following aims:

- To determine which E6 carboxy-terminal residues are critical for both, PDZ and kinase recognition.

- To identify if there is a correlation between the functional flexibility of E6 and phosphorylation within the PBM.

- To Identify additional kinases that could phosphorylate E6 *in vitro*

- To assess the importance of possible phosphorylatable residues upstream of the PBM, and if this correlates with carcinogenicity

- To determine how phosphorylation outside the PBM affects the function of E6

- To determine in non-cancer-causing E6 proteins (i.e HPV-11 E6), could have a carcinogenic effect after DNA damage stimuli.

HYPOTHESIS

Part 1 and Part 2:

HPV E6 evolution has been linked to its functional flexibility. The generation of new interactions has allowed high-risk HPV types to adapt to new niches in the cervical epithelia. The more interaction partners an E6 protein has, its carcinogenic potential increases. Likewise, phosphorylation of E6 within the PBM, is considered a mechanism of regulation that may facilitate the interactions with specific binding partners important for specific functions like transport, localisation and degradation.

Indeed, both HPV-18 and HPV-16 are the most carcinogenic types, and this correlates with their capacity to be singly and multiply phosphorylated, both within and outside the PBM, possibly in sites important for binding different proteins individually or in a complex.

Part 3:

Although low-risk HPV types are generally not carcinogenic and do not possess a PBM, they maintain the ability to replicate very efficiently in the epithelium and cause disease. Since the main oncogenic function of high-risk types is to bind p53 and degrade it, the low-risk types' capacity to bind but not degrade p53 has been an enigma, suggesting that p53 might have had a function important to HPV before high-risk types evolved to degrade it; or that p53 degradation was a controlled event, dependent on p53 status and the cellular environment; this possibility is supported by the inherent ability of the E6-E6AP complex to degrade E6’s targets.

CHAPTER 2: MATERIALS AND METHODS

Cell culture and transfection

HaCat, H1299 and wild type HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/ml), and glutamine (300 µg/ml). All the cell lines were cultured at 37°C with 5% CO_2.

H1299 and HEK293 cells were transfected using the calcium phosphate precipitation method (Wigler et al., 1979), and harvested after 16, 24 or 48 hours, depending on the cell type and the protein used.

Plasmids, cloning and mutagenesis

The glutathione S-transferase fusion proteins were previously cloned in pGEX2T plasmids. The vectors expressing GST, GST-HPV-18E6, GST-HPV-16E6 have been previously described (Boon et al., 2015; Boon & Banks, 2013).
The GST-HPV-66E6 and GST-HPV40E6 plasmids were kindly provided by Miranda Thomas (Thomas et al 2016). The GST-HPV-66E6 A150R, GST-HPV-66E6 T151R, GST-HPV-66E6 A150R/ T151R and GST-HPV-40 C154V mutants were generated were generated using an adapted site-directed mutagenesis PCR method.
The pCDNA3.1-HA-Scrib, pGW1-HA-Dlg and pCDNA3.1-Flag-MAGI-1 expression plasmids have been described previously (Gardiol et al., 2002; Kranjec et al., 2014; Kranjec et al., 2016). The pCDNA3.1-16E6 were provided by Boon SS. The pcDNA3.1 FLAG-p53 (Pim et al., 1997), pGWI:HA-Dlg (Gardiol et al., 2002), and pGW1-HA-16E6 was provided by Ron Javier.
The pcDNA3.1-wild type p53, pcDNA3.1 S15A p53 and pcDNA3.1 S15D p53 were previously described (Loughery et al., 2014) and obtained through Addgene.

All GST-HPV-18E6, GST-HPV-66E6, GST-HPV-40E6 and HPV-16E6; and the pcDNA3.1-HA-HPV-16E6 and pGW1-HA-HPV-16E6 substitution and deletion mutants were generated using an adapted site-directed mutagenesis PCR method (Carey et al., 2013), using Taq Platinum polymerase (Promega). Resulting plasmid were all verified by DNA sequencing (Eurofins).

All mutants generated and primers used are shown in Table 4.

HPV type	Plasmid backbone
HPV-18 E6 wt	
18_R152A Fw - CAGGAACGACTCCAAgcACGCAGAGAAACACAA 18_R152A Rv - TTGTGTTTCTCTGCGTgcTTGGAGTCGTTCCTG 18_R153A Fw - GAACGACTCCAACGAgcCAGAGAAACACAAGTA 18_R153A Rv - TACTTGTGTTTCTCTGgcTCGTTGGAGTCGTTC 18_R154A Fw - CGACTCCAACGACGCgcAGAAACACAAGTATAA 18_R154A Rv - TTATACTTGTGTTTCTgcGCGTCGTTGGAGTCG 18_V158C Fw - CGACGCAGAGAAACACAAtgtTAATATTAAGTATGCAT 18_V158C Rv - ATGCATACTTAATATTAacaTTGTGTTTCTCTGCGTCG	pGEX2T-GST
HPV-40 E6 wt	
40_C154V Fw - CGCTCGGAGACATTGgtTTAAGAATTCGGTACC 40_C154V Rv - GGTACCGAATTCTTAAacCAATGTCTCCGAGCG	pGEX2T-GST
HPV-66 E6 wt	
66_A150R Fw - CATACGAGTAGACAAcgTACAGAATCTACAGTA 66_A150R Rv - TACTGTAGATTCTGTAcgTTGTCTACTCGTATG 66_T151R Fw - CATACGAGTAGACAAGCTcgAGAATCTACAGTA 66_T151R Rv - TACTGTAGATTCTcgAGCTTGTCTACTCGTATG 66_A150R/T151R Fw - CATACGAGTAGACAAcgTcgAGAATCTACAGTA 66_A150R/T151R Rv - TACTGTAGATTCTcgAcgTTGTCTACTCGTATG	pGEX2T-GST
HPV-16 E6 wt 16_dPBM Fw - CAGATCATCAAGAACACGTAGATAATCATGCATGGAGATACACC 16_dPBM Rv - GGTGTATCTCCATGCATGATTATCTACGTGTTCTTGATGATCTG	pGEX2T-GST
HPV-16 E6 wt	
16_T156A Fw - AGAACACGTAGAGAAGCCCAGCTGTAATCATGC 16_T156A Rv - GCATGATTACAGCTGGGCTTCTCTACGTGTTCT 16_T156A Fw_II - caAGAACACGTAGAGAAgCCCAGCTGTAAGAATTC 16_T156A Rv_II - GAATTCTTACAGCTGGGcTTCTCTACGTGTTCTtg	pGEX2T-GST pGW1-HA

HPV-16 E6 wt	
16_T152A Fw - TTGTTGCAGATCATCAAGAgCACGTAGAGAAACCCAGCT 16_T152A Rv - AGCTGGGTTTCTCTACGTGcTCTTGATGATCTGCAACAA 16_T152D Fw - GTTGCAGATCATCAAGAgatCGTAGAGAAACCCAGCT 16_T52D Rv - AGCTGGGTTTCTCTACGTtcTCTTGATGATCTGCAAC 16_T152E Fw - GTTGCAGATCATCAAGAGAACGTAGAGAAACCCAGCT 16_T152E Rv - AGCTGGGTTTCTCTACGTtcTCTTGATGATCTGCAAC	pGEX2T-GST pcDNA3.1-HA pGW1-HA

HPV-16 E6 wt		
16_S145A Fw - GACCGGTCGATGTATGgCTTGTTGCAGATCATC 16_S145A Rv - GATGATCTGCAACAAGcCATACATCGACCGGTC 16_S149A Fw - GTATGTCTTGTTGCAGAgCATCAAGAACACGTAGA 16_S149A Rv - TCTACGTGTTCTTGATGcTCTGCAACAAGACATAC 16_S150A Fw - GTCTTGTTGCAGATCAgCAAGAACACGTAGAGA 16_S150A Rv - TCTCTACGTGTTCTTGcTGATCTGCAACAAGAC 16_S145D Fw - GACCGGTCGATGTATGgaTTGTTGCAGATCATCA 16_S145D Rv - TGATGATCTGCAACAAtcCATACATCGACCGGTC 16_S149D Fw - GTATGTCTTGTTGCAGAgatTCAAGAACACGTAGAGA 16_S149D Rv – TCTCTACGTGTTCTTGAatcTCTGCAACAAGACATAC 16_S150D Fw - TGTCTTGTTGCAGATCAgatAGAACACGTAGAGAAAC 16_S150D Rv - GTTTCTCTACGTGTTCTatcTGATCTGCAACAAGACA	pcDNA3.1-HA pGW1-HA	pGEX2T-GST

HPV-16 E6 wt	S149; S150	
16_SSAA Fw - GTATGTCTTGTTGCAGAgCAgCAAGAACACGTAGAGA 16_SSAA Rv - TCTCTACGTGTTCTTGcTGcTCTGCAACAAGACATAC 16_SSDD Fw - GTATGTCTTGTTGCAGAgacgacAGAACACGTAGAGAAAC 16_SSDD Rv - GTTTCTCTACGTGTTCTgtcgtcTCTGCAACAAGACATAC		pGEX2T-GST pcDNA3.1-HA pGW1-HA

HPV-16 S149A; S150A	S145	pGEX2T-GST
16_SSS_3A Fw - GACCGGTCGATGTATGgCTTGTTGCAGAGCAGC 16_SSS_3A Rv - GCTGCTCTGCAACAAGcCATACATCGACCGGTC		pcDNA3.1-HA pGW1-HA

HPV-16 S149D; S150D	S145	pGEX2T-GST
16_SSS_3D Fw - CCGGTCGATGTATGgaTTGTTGCAGAgacgac 16_SSS_3D Rv – gtcgtcTCTGCAACAAtcCATACATCGACCGG		pcDNA3.1-HA pGW1-HA

Table 4.- Mutagenesis primers.- All mutants were generated by site-directed mutagenesis. Each primer sequence shown is in the 5' to 3' orientation.

GST fusion protein production and purification

All the expression constructs were transformed into *E. coli* strain DH5-α, and GST fusion proteins were expressed and purified as described previously (Thomas et al., 1996). Briefly, the clones were grown in 40ml of Luria Broth (LB) culture media containing 75µg/ml Ampicillin (Sigma) overnight at 37°C. The overnight bacterial cultures were transferred into 400ml of LB culture media containing 75µg/ml Ampicillin and incubated at 37°C for 2 hours. The recombinant protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) to a final concentration of 1mM and further incubated for 3 to 4 hours at 37°C. For low producing clones, like GST-HPV-66 E6 and its mutants, the induction was carried out at room temperature overnight or at 30°C overnight. After incubation, the bacteria were harvested at 5000 x g for 10 minutes. The supernatant was discarded, and the pellets were lysed with 10ml ice cold PBS containing 1.5% Triton X-100 and sonicated twice for 20 seconds at 80% amplitude. The sonicated lysates were then centrifuged at 16000 x g for 15 minutes. The supernatants were collected and incubated with glutathione-conjugated agarose beads (Sigma) on a rotating wheel for 1 hour at 4°C. The GST-fusion protein-bound beads were then centrifuged at 1000 x g for 3 minute and the supernatant was discarded. The beads were washed thrice with PBS containing 1.5% Triton X-100. Subsequently, the GST-fusion protein-bound beads were stored in 1XPBS containing 1.5% Triton X-100 and 40% glycerol at -20°C for long term storage.

In vitro phosphorylation assays

For each phosphorylation assay, the GST fusion proteins were washed three times with the respective kinase buffers containing 0.1% NP-40. The specific protocols and buffers used are as follows:

AKT phosphorylation assays: 0.2µg of AKT1 kinase was used in 1X reaction buffer A from the AKT1 kinase enzyme system (Promega), diluted in AKT kinase buffer, containing 25mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, and 5mM dithiothreitol (DTT), and incubated with 2.5µCi radiolabeled [ɣ-32P] ATP. The reaction was carried out at 30°C for 35 min.

Chk1 phosphorylation assays: 0.2µg of CHK1 was used in 1X reaction buffer A (Promega) from the CHK1 kinase assay system (Promega), diluted in CHK1 kinase buffer, containing 25mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, and 5mM dithiothreitol (DTT) and incubated with 2.5µCi radiolabeled [ɣ-32P] ATP. The reaction was carried out at room temperature for 1 hour according to the manufacturer's instructions.

PKA phosphorylation assay: The GST fusion proteins were incubated in 20µl of PKA kinase

buffer with 2.5 µCi radiolabeled [ɣ-32P] ATP and cAMP-dependent protein kinase catalytic subunit (Promega) for 20 minutes at 30°C. The PKA buffer contained 25mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, and 70mM NaCl.

PKC phosphorylation assay: The GST fusion proteins were incubated in 20µl PKC kinase buffer with 2.5µCi radiolabeled [ɣ-32P] ATP, 100µM ATP and 25U purified PKC, consisting primarily of α, β and δ isoforms, and, to a lesser extent, γ and ζ isoforms (Promega); for 30 minutes at 30°C. The PKC buffer contained 20mM Hepes (pH 7.4), 1.67mM $CaCl_2$, 1mM DTT and 10mM $MgCl_2$.

CK1 phosphorylation assay: The GST fusion proteins were incubated at 30°C for 35 minutes in 20µl 1X CK1 reaction buffer (NEB) with 2.5µCi radiolabeled [ɣ-32P] ATP, 200µM ATP and 30U casein kinase I truncated monomer of the CK1δ isoform, which lacks the carboxy-terminal regulatory domain (NEB).

GSK3β phosphorylation assay: The GST fusion proteins were incubated at 30°C for 35 minutes in 20µl of 1X PK NEBuffer™ for Protein Kinases (NEB) with 2.5µCi of radiolabeled [ɣ-32P] ATP, 200µM ATP and 30U purified rabbit GSK3β (NEB).

All reactions were then washed with the respective kinase buffers containing 0.1% NP-40 and analyzed by SDS-PAGE and autoradiography. For CK1 and GSK3β, the washing buffer contained 25mM Tris HCl (pH 7.5), 10mM MgCl2 and 0.1% NP-40.

Peptide pulldown assays

500ug lyophilized peptide was resuspended in RGMT lysis buffer (50mM HEPES (pH 7.4), 150mM NaCl, 1mM $MgCl_2$, 1% Triton-X-100) to a final concentration of 10mg/ml and bound to streptavidin-conjugated magnetic sepharose beads (Streptavidin-MagSepharose, GE Healthcare) by incubation at 4°C on a rotating wheel for 1 hour, then washed three times with RGMT lysis buffer.

The HaCat or HEK293 cells were lysed with RGMT buffer with 1X protease inhibitor cocktail I (Calbiochem), and 5mM of the inhibitor of phosphoseryl and phosphothreonyl phosphatases, Sodium Fluoride (NaF), in the case of experiments using phosphorylated peptides. Subsequently the lysed cells were collected by scraping and centrifugation at 16000 x g for 10 minutes. The supernatant, containing the soluble proteins of the cell extract, was then pre-cleared by incubation at 4°C on a rotating wheel for 1 hour with empty streptavidin-conjugated magnetic beads. After removal of the pre-clearing beads, the cell extract was incubated at 4°C on a rotating wheel for 2 hours with each of the biotinylated peptides bound to streptavidin-

conjugated magnetic sepharose beads. The beads were washed three times with RGMT lysis buffer without protease inhibitors, transferred to fresh tubes and washed once more with RGMT lysis buffer without Triton-X-100, and resuspended in 2X SDS sample buffer (160mM Tris-HCl (pH6.8), 1.6% SDS, 0.2% (w/v) bromophenol blue, 50% glycerol, and 16mM β-mercaptoethanol) for SDS-PAGE and western blot analysis.
In the case of peptide binding assays for mass spectrometry analysis. The beads were washed three times with RGMT lysis buffer without TritonX-100 (RGM buffer). Then 5% of the beads was taken for western blot analysis and the remainder was subjected to trypsin-digest and the products analysed by mass spectrometry.

Sample preparation for mass spectrometry

The proteins were eluted directly from the beads using 50 ng of sequencing grade trypsin (Promega) in 20 nM diammonium phosphate pH 8.0 for 6 hours at 37°C. The supernatant was removed from the beads and the cysteines were reduced and alkylated by boiling for 2 minutes in the presence of 10 nM Tris (2-carboxyethyl) (Pierce, Milan, Italy), followed by incubation with 20mM acetaminophen (Sigma) for 1 hour at 37°C. The reaction was stopped by adding acetic acid (Sigma) to 0.1%. The final mixture was desalted using C18 Ziptips (Millipore, Milan, Italy) and lyophilized to dryness (Tomaić et al., 2009).

Mass Spectrometry

Nanobore columns were constructed using Picofrit columns (NewObjective, Woburn, MA, USA) and packed with 15 cm of 1.8 mm Zorbax XDB C18 particles using homemade high-pressure column loader. The desalted samples were injected onto the nanobore column in buffer A (10% methanol/ 0.1% formic acid) and the column was eluted with a discontinuous gradient and sprayed directly into the LTP ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA) (Tomaić et al., 2009).

GST-binding assays

Transiently transfected HEK293 cells were collected and lysed in E1A buffer (50mM Hepes (pH 7.0, 250mM NaCl, 0.1% NP-40, with protease and phosphatase inhibitors) following by gentle syringing and incubation on ice for 30 minutes. The cell lysates were then centrifuged at 16000 x g for 10 minutes. A similar procedure was used for HaCat cell extraction, but using RGMT

buffer instead. HaCat cells were used for analyzing binding of endogenous proteins. The GST-complexes were washed three times with E1A buffer without protease inhibitors and resuspended in 2X SDS sample buffer for SDS-PAGE and western blot analysis.
For indirect binding assays, the cell extracts were incubated with the purified GST-fusion proteins and incubated at 4°C or room temperature, depending on the proteins analysed, for 2 hours. All incubations were done using the rotation wheel.

Half-life experiments

At 24- or 48-hours post transfection, the cells were treated with cycloheximide (50ug/ml in dimethyl sulfoxide-DMSO) to block protein synthesis at different time points. DMSO alone-treated cells were used as the control (or untreated-UT cells). Cells were then lysed and collected in SDS sample buffer and analysed by western blotting.
The intensity of the bands on the X-ray films was quantified using the ImageJ program. The standard deviation was calculated based on a minimum of three independent experiments.

p53 *In vivo* degradation assays

For *in vivo* degradation assays, H1299 cells were transiently transfected with plasmids expressing Flag-p53 and untagged p53, S15D p53 and S15D p53 in the presence and absence of HA-tagged HPV-11 E6, at a ratio 3:1. As transfection efficiency controls, the cells were co-transfected with LacZ control vector. After 24 hours the cells were treated with Etoposide overnight. For subsequent half-life experiments, the cells were additionally treated with cycloheximide, as described before. The cells were collected in 2X SDS sample buffer and analyzed by SDS-PAGE and western blotting.

Western blotting and antibodies

Immunoprecipitated protein or total cell extracts were obtained by lysing the cells directly with 2X SDS sample buffer. Western blotting and processing were done as described previously (Massimi et al 2008). Briefly, the nitrocellulose membrane (blot) was blocked in 10% milk in PBS/0.5% Tween20 (PBST) for 1 hour at 37°C. For certain antibodies, the blocking solution was 1X Tris-buffered saline (50mM Tris-HCl (pH 7.5, 150mM NaCl) (TBS)/0.1% Tween20 (1x TBST) instead of PBST, or 5% milk or 3% Bovine serum albumin-BSA instead of 10% milk. The subsequent primary and secondary incubations used the same conditions as the blocking.
The primary antibodies were diluted in PBST and incubated on the blot at room temperature for

1 hour or at 4°C overnight, with gentle rocking. After washing three times with PBST, the blot was incubated for 1 hour at room temperature with the corresponding secondary antibodies conjugated to horseradish peroxidase (HRP, Dako), diluted (1:2000) in PBST/10% milk (Table 5). After three washes of 10 minutes each with gentle rocking, the blot was developed using the ECL detection system (GE Healthcare) according to the manufacturer's protocol.

The following antibodies were used: mouse monoclonal anti-β-galactosidase (Promega), mouse monoclonal anti-HA (Roche), mouse monoclonal anti-p53 DO-1 (Santa Cruz); mouse monoclonal anti-SAP97 (Santa Cruz), mouse monoclonal anti-FLAG M2 (Sigma), mouse monoclonal anti-HA-peroxidase (Sigma-Aldrich), mouse monoclonal anti-GFP, goat monoclonal anti-Scrib (Santa Cruz), rabbit monoclonal anti-PKCα (Santa Cruz), mouse monoclonal anti-syntrophin-β2 (Sigma-Aldrich), rabbit monoclonal anti-Tax1BP3 (Sigma), mouse monoclonal anti-RACK1 (Santa Cruz), mouse monoclonal anti-PKCα (Santa Cruz). All concentrations and incubations conditions used, and the catalogue numbers are described in Table 5.

Immunofluorescence assays

HEK293 and H1299 cells were seeded at a density of approximately 1.2 x 10^5 cells on glass coverslips, allowed to attach overnight then transfected or treated as appropriate. The cells were washed with PBS and fixed using 4% paraformaldehyde for 20 minutes and permeabilized in PBS/ 0.1% Triton X-100 for 5 minutes, or PBS/ 0.2% saponine and 3% BSA, for 30 minutes; depending on the antibody used (Table 5). After extensive washing with PBS, the cells were incubated with 100mM glycine for 10 minutes, washed again and immunostained with the appropriate antibodies diluted in PBS alone or PBS containing 0.01% saponine and 0.2% BSA. The incubation was done at 37°C for 2 hours in a humidified chamber. The coverslips were then washed thrice with PBS and incubated with the appropriate fluorophore-conjugated secondary antibodies in the same buffer as the primary antibody (Table 5), for 30mins at 37°C in a humidified chamber. The coverslips were washed thrice with PBS and incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1:25 000) for 5 seconds for nuclear staining and washed twice with distilled water before being mounted onto glass slides. The images were taken using a Carl Zeiss LSM510 META confocal microscope. The co-localizations were quantified using ImageJ, and the Pearson's Correlation Coefficient (PCC) calculated for individual cells in each set of images, where values above the mean of the wild type control indicate higher degrees of colocalization. Furthermore, the cytoplasmic:nuclear (C:N) ratio was calculated based on the wild type average C:N ratio of 0.5 and compared with the ratio of the respective mutants. Ratios

lower than 0.5 indicate nuclear retention and ratios higher than 0.5 indicate cytoplasmic retention.

WB Primary Antibodies				
Antibody name	Dilution	WB Incubation conditions	Company	Catalogue number
p53 Antibody (DO-1)	1:5000	1XPBST-10%milk	Santa Cruz	sc-126
SAP 97 Antibody (2D11)	1:500	1XPBST-10%milk	Santa Cruz	sc-9961
Scrib Antibody (C-6)	1:500	1XPBST-10%milk	Santa Cruz	sc-55543
Anti-β-galactosidase antibody	1:5000	1XPBST-10%milk	Promega	Z3783
GFP antibody (B-2)	1:1000	1XPBST-10%milk	Santa Cruz	cs-9996
Anti-HA (12CA5)	1:500	1XPBST-10%milk	Roche	11583816001
Anti-Tax1BP3	1:500	1XPBST-3%BSA	Sigma	HPA046410
Monoclonal anti-HA peroxidase	1:500	1XPBST-10%milk	Sigma	H6533
SNTB2 Monoclonal Antibody (1351)	1:500	1XPBST-3%BSA	Invitrogen	MA1-745
PKCα Antibody (C-20)	1:1000	1XPBST-3%BSA	Santa Cruz	sc-208
RACK1 Antibody (B-3)	1:4000	1XPBST-10%milk	Santa Cruz	sc-17754
WB Seconday Antidodies				
Antibody name	Dilution	WB Incubation conditions	Company	Catalogue number
Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP	1:2000	1XPBST-10%milk	Dako	P0260
Polyclonal Goat Anti-Mouse Immunoglobulins/HRP	1:2000	1XPBST-10%milk	Dako	P0446
Polyclonal Rabbit Anti-Goat Immunoglobulins/HRP	1:2000	1XPBST-10%milk	Dako	P0449
IF Primary Antibodies				
Antibody name	Dilution	IF Incubation conditions	Company	Catalogue number
p53 Antibody (DO-1)	1:50	1XPBS/0.01% Saponine - 0.2%BSA	Santa Cruz	sc-126
Anti-HA (12CA5)	1:100	1XPBS/0.01% Saponine - 0.2%BSA	Roche	11583816001
IF Seconday Antidodies				
Antibody name	Dilution	IF Incubation conditions	Company	Catalogue number
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:600	1XPBS/0.01% Saponine - 0.2%BSA	Invitrogen	A-21202
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	1:600	1XPBS/0.01% Saponine - 0.2%BSA	Invitrogen	A-21207

Table 5.- Western blotting and Immunofluorescence antibodies.- Primary and secondary antibodies used for Western blot and Immunofluorescence experiments.

Inhibitors, chemicals and reagents

The inhibitors and chemicals used to treat H1299 and HEK293 cells were 500µM hydrogen peroxide (H_2O_2) solution for 5 hours; 100µg/ml Cycloheximide, for a maximum of 2 hours; 200ng/ml Nocodazole, for 16 hours; 2.5mM Thymidine (Sigma Aldrich), for 16 hours. The chemotherapeutic reagents used were: 5µM Teniposide, for 16 hours and 10µM Etoposide, for 16 hours (both, Sigma Aldrich). The concentrations of all the above chemicals were optimized based on the cell toxicity and their efficacy for each cell line.

Statistical Methods

All experiments were performed a minimum of three times, and data are shown as mean and standard error of mean. Statistical significance was calculated using the GraphPad prism software. To compare two groups the paired Student's t-test was performed. A p-value below 0.05 was considered statistically significant and throughout the p-values have been defined as follows *p<0.05, **p<0.001, ***p<0.0001, while "ns" represents a non-significant p-value above 0.05.

To obtain the percentage of relative abundance of a protein in western blot experiments, the X-ray films were scanned, and the intensity of the bands and the background was measured using ImageJ (FiJi) software. The final relative quantification values are the ratio of the studied protein's net band intensity: the loading control protein's net band intensity. The percentages are obtained taking the input controls, the "time-zero controls" (for half-life experiments), and the wild-type (for GST-binding assays), as 100%. All experiments were performed at least three times, and data are shown as mean and standard error of mean in all graphs.

CHAPTER 3: RESULTS

Part 1: Acquisition of a Phospho-acceptor Site Enhances HPV E6 PDZ Binding Motif Functional Promiscuity.

The PBM allows high-risk E6 proteins to bind cellular PDZ domain-containing targets, mainly through the last four consensus residues, although the PDZ proteins can recognize up to the last nine residues at the carboxyl terminus of the E6 PBM. Indeed, the differences in the amino acid sequence of the core and upstream of the canonical PBM, greatly affects the spectrum of PDZ binding partners of E6 (Thomas et al., 2005, 2016), and correlates with the HPV risk classification (groups 1, 2A, 2B and 3). Indeed, the HR types from Group 1 have the most promiscuous E6 proteins, while the other possibly or probably carcinogenic and low-risk type HPV-40, have a more restricted PDZ target range (Thomas et al., 2016).

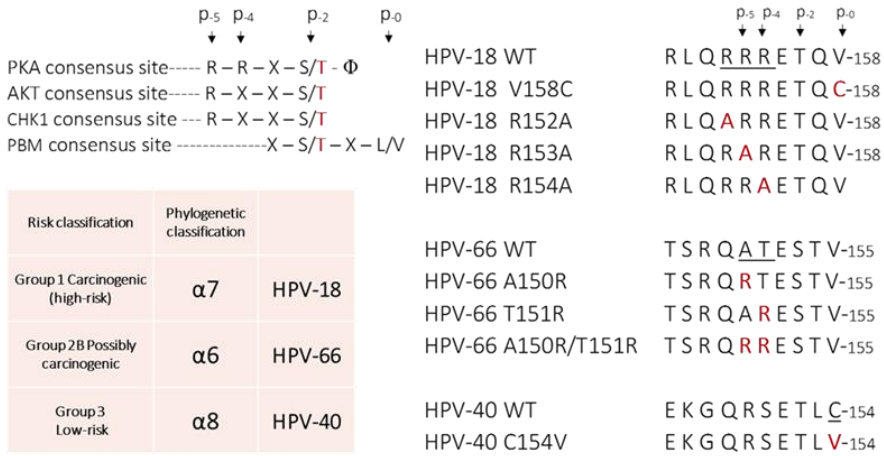
Furthermore, the phospho-acceptor site embedded within the PBM, adds to the functional diversity of the E6, negatively regulating the interaction with PDZ targets (hDlg1, MAGI-1, hScrib) and creating alternative associations with members of the cellular 14-3-3 protein family (Boon et al., 2015; Boon & Banks, 2013; Delury et al., 2013a; Thatte et al., 2018). Accordingly, the identity of the kinase involved (PKA, AKT or CHK1) depends on the composition of the PBM sequence.

To understand whether the ability of HPV E6 oncoproteins to target multiple PDZ domain-containing substrates is potentially linked with the acquisition of a phospho-acceptor site within the PDZ binding motifs (PBMs), we generated mutants of the core and upstream PBMs of the carcinogenic HPV-18 from high-risk group 1 viruses; HPV-66 from the possibly carcinogenic group 2B and HPV-40 from low-risk group 3; and assessed their phosphorylation status with respect to known kinases AKT, PKA and CHK1. In addition, we evaluated wild type and mutant PBM peptides and the pattern of PDZ domain-containing substrates that are bound when those residues that comprise the kinase recognition motif are mutated. We have found that there is a very close correlation between the residues critical for phosphorylation and the ability to bind to multiple PDZ domain containing proteins.

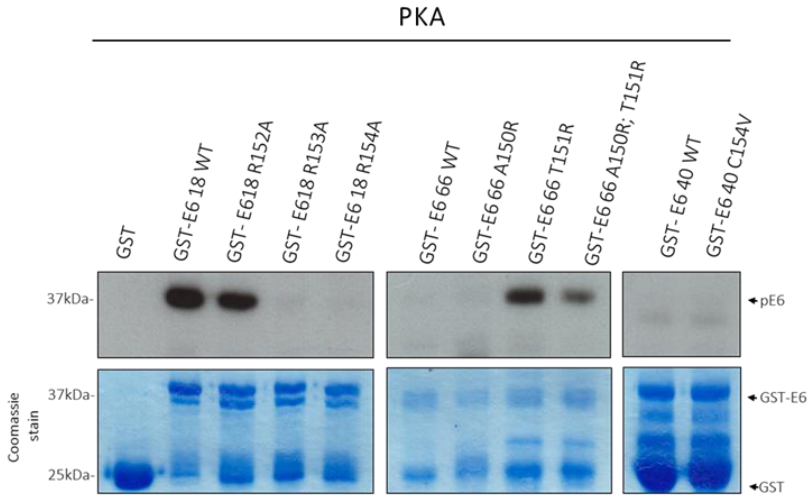
HPV-18, -66 and -40 E6 proteins from different risk classification groups have diverse phosphorylation patterns.

Knowing that HPV-18 E6 is phosphorylated by PKA, AKT and CHK1 kinases, we compared the amino acid sequences of HPV-66 and HPV-40 PBMs with the recognition motif sequences of PKA, AKT and CHK1 kinases (Figure 12a). As can be seen, HPV-18 E6 has a very strong consensus recognition site for all three kinases, HPV-66 and HPV-40 E6 are quite distinct. However in order to directly assess their respective susceptibility to be phosphorylated by the different kinases we performed a series of *in vitro* kinase assays using purified kinases AKT, PKA and CHK1; and purified HPV-E6 glutathione S-transferase (GST) fusion proteins as substrates in the presence of radiolabeled ATP. The results presented in Figures 12b, c and d show that HPV-18 E6 is strongly phosphorylated by PKA and, to a somewhat lesser extent, by AKT and CHK1, as expected. However, neither the HPV-66 nor HPV-40 E6 proteins are phosphorylated by either PKA, AKT or CHK1 (Figure 12b-d). This was not surprising for HPV-66 since it lacks sequences that would predict phosphorylation by any of these kinases, however HPV-40 E6 does possess sequences which might have predicted phosphorylation by either AKT or CHK1.

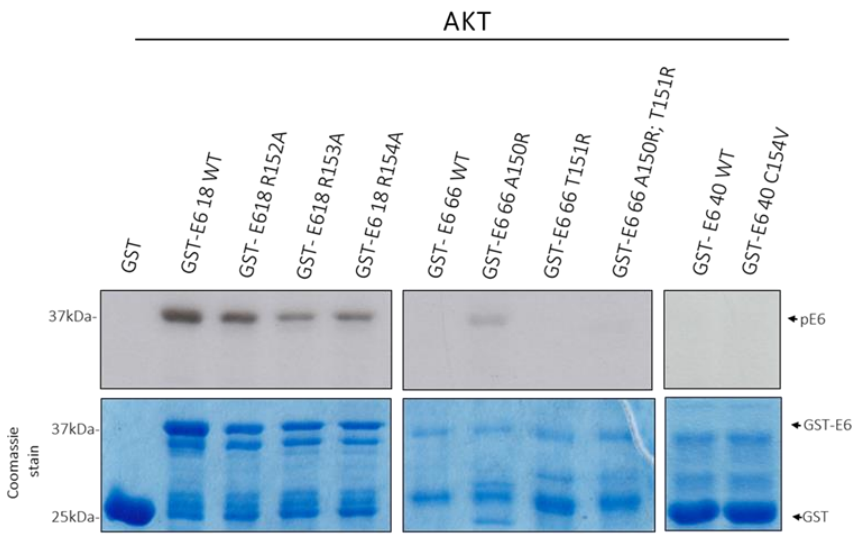
a.


p-5 p-4 p-2 p-0
PKA consensus site----- R – R – X – S/T - Φ
AKT consensus site----- R – X – X – S/T
CHK1 consensus site --- R – X – X – S/T
PBM consensus site ----------X – S/T – X – L/V
Risk classification
Phylogenetic classification
Group 1 Carcinogenic (high-risk)
α7
HPV-18
Group 2B Possibly carcinogenic
α6
HPV-66
Group 3 Low-risk
α8
HPV-40
HPV-18 WT R L Q R R R E T Q V-158
HPV-18 V158C R L Q R R R E T Q C-158
HPV-18 R152A R L Q A R R E T Q V-158
HPV-18 R153A R L Q R A R E T Q V-158
HPV-18 R154A R L Q R R A E T Q V
HPV-66 WT T S R Q A T E S T V-155
HPV-66 A150R T S R Q R T E S T V-155
HPV-66 T151R T S R Q A R E S T V-155
HPV-66 A150R/T151R T S R Q R R E S T V-155
HPV-40 WT E K G Q R S E T L C-154
HPV-40 C154V E K G Q R S E T L V-154

b.


PKA
GST
GST-E6 18 WT
GST- E618 R152A
GST- E618 R153A
GST-E6 18 R154A
GST- E6 66 WT
GST-E6 66 A150R
GST-E6 66 T151R
GST-E6 66 A150R; T151R
GST- E6 40 WT
GST-E6 40 C154V
37kDa-
pE6
Coomassie stain
37kDa-
GST-E6
25kDa-
GST

c.


AKT
GST
GST-E6 18 WT
GST- E618 R152A
GST- E618 R153A
GST-E6 18 R154A
GST- E6 66 WT
GST-E6 66 A150R
GST-E6 66 T151R
GST-E6 66 A150R; T151R
GST- E6 40 WT
GST-E6 40 C154V
37kDa-
pE6
Coomassie stain
37kDa-
GST-E6
25kDa-
GST

d.

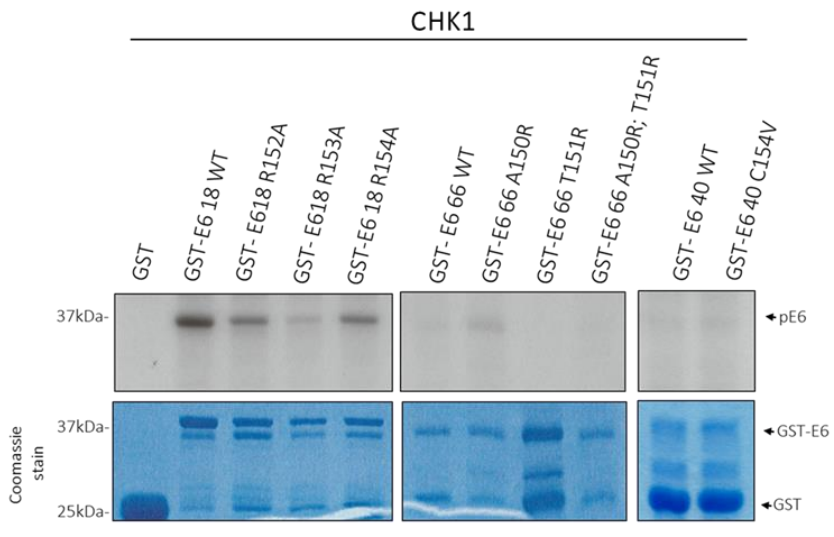

CHK1
GST
GST-E6 18 WT
GST- E618 R152A
GST- E618 R153A
GST-E6 18 R154A
GST- E6 66 WT
GST-E6 66 A150R
GST-E6 66 T151R
GST-E6 66 A150R; T151R
GST- E6 40 WT
GST-E6 40 C154V
37kDa-
pE6
Coomassie stain
37kDa-
GST-E6
25kDa-
GST

Figure 12. HPV E6 PBMs are differentially phosphorylated by different kinases *in vitro*. (a) The HPV E6 PBM sequences and the AKT, PKA and CHK consensus recognition sequences, showing the positions p0, p-2, p-4 and p-5. The panel shows wild-type and mutant carboxyl-terminus sequences of the E6 proteins analysed. The wild-type amino acids are underlined and the mutations are shown in red. (b) In vitro phosphorylation by purified PKA of wild-type and mutant GST-E6 proteins from HPV-18, -66 and -40, in the presence of γ[32]-ATP. (c) In vitro phosphorylation by purified AKT of wild-type and mutant GST-E6 proteins from HPV-18, -66 and -40, in the presence of γ[32]-ATP. (d) In vitro phosphorylation by purified CHK1 of wild-type and mutant GST-E6 proteins from HPV-18, -66 and -40, in the presence of γ[32]-ATP.

Sequential amino acid alterations in upstream residues in HPV-66 E6 can allow phosphorylation by PKA.

In order to understand which residues within the E6 PBM sequences were essential for phosphorylation we generated a number of amino acid substitutions within HPV-18, HPV-66 and HPV-40 E6 proteins, and then monitored their ability to be phosphorylated by the different kinases. As can be seen from Figure 12B-D, the wild-type HPV-66 E6 protein is not phosphorylated by either PKA, AKT or CHK1, and mutating the alanine in position p-5 to an arginine (A150R) does not appear to affect PKA phosphorylation (Figure 12b), but it does permit very low levels of AKT and CHK1 phosphorylation (Figure 12c-d). In contrast, the arginine residue introduced at position p-4 (T151R) increases the phosphorylation by PKA, but not by AKT or CHK1. In addition, the A150R/T151R double mutant is also phosphorylated by PKA and not by AKT, with the T151R mutant being the more intensely phosphorylated. To corroborate the importance of these two p-4 and p-5 arginine residues immediately upstream of the PBM for PKA phosphorylation, we mutated each of the three arginine residues upstream of the HPV-18 E6 PBM, generating the mutants R152A, R153A and R154A. As shown in Figure 12B, phosphorylation of HPV-18 E6 by PKA is strongly reduced when the arginine residues in positions p-4 and p-5 are mutated. Additionally, Figures 12C and 12D also show that the HPV-18 R153A mutant has reduced CHK1 and AKT phosphorylation, but this residue does not appear to be as critical as in the case of PKA. Interestingly, the HPV-66 E6 T151R mutant and HPV18 E6 R153A mutant have the same residues (AR) at the p-4 and p-5 positions, but show

different PKA phosphorylation profiles, suggesting that other residues, such as the p-3 serine or the extra p-2 threonine of the HPV-66 E6 PBM, might favor phosphorylation by PKA (Figure 12b).

We were also intrigued by the lack of phosphorylation in HPV-40 E6, despite the presence of an upstream arginine which might be predictive of phosphorylation by AKT or CHK1. However, HPV-40 E6 also has a major alteration in the presence of a carboxy terminal cysteine instead of valine. To determine whether this residue might be having a negative influence we made a single amino acid substitution HPV-40 E6 C154V and then analysed the effect on phosphorylation. As can be seen from Figure 12b-d, this has no effect on phosphorylation by either PKA, AKT or CHK1. Taken together these results indicate a complex pattern of regulation of E6 phosphorylation, with multiple upstream arginine residues appearing to be required for optimal levels of phosphorylation, and in the case of HPV-40 E6, the downstream cysteine also acting in a negative fashion.

Residues upstream of the PBM affecting phosphorylation also affect the interaction with hDlg1, MAGI-1 and hScrib PDZ proteins.

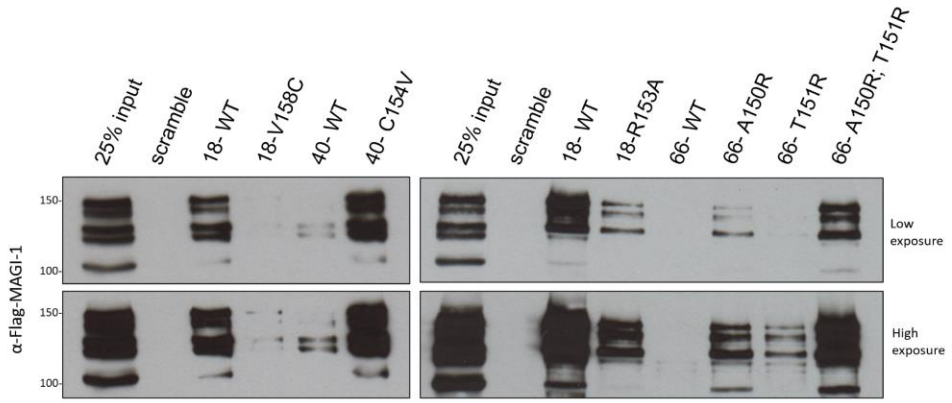
After analyzing their susceptibility to phosphorylation, we wanted to evaluate whether alterations in the PBMs of HPV-18, HPV-40 and HPV-66, shown in Figure 12a, changed the capacity of the PBMs to associate with the known PDZ domain-containing targets hDlg1, hScrib and MAGI-1. We used biotin-conjugated peptides corresponding to the last ten amino acids of the E6 proteins from HPV-18, HPV-40 and HPV-66, as shown in Figure 12a, to analyze the effect of mutations that change the PKA, AKT and CHK1 recognition sequences upon the ability of the peptides to pull down these well-characterized PDZ domain-containing targets of HPV-18 E6. The biotinylated peptides were conjugated to streptavidin magnetic beads, and then were further incubated with extracts of HEK293 cells previously transfected with plasmids expressing the different PDZ domain-containing proteins. Subsequently, the bound proteins were detected by western blotting. The results with MAGI-1 presented in Figure 13 show that wild-type HPV-18 PBM interacts very strongly with MAGI-1, whilst HPV-40 and HPV-66 are extremely weak, and this is largely in agreement with previous studies (Muench et al., 2009; Miranda Thomas et al., 2016; Van Doorslaer et al., 2015). However, the critical role of the C/V substitution is clearly apparent, since the HPV-18 V158C mutation destroys MAGI-1 recognition, whilst the HPV-40 C154V greatly enhances MAGI-1 binding (Figure 13).

Additionally, the HPV-18 R153A mutant, which has decreased phosphorylation by PKA and CHK1, concomitantly greatly decreases MAGI-1 recognition. Conversely, the single and double arginine mutants of HPV-66 significantly promote MAGI-1 recognition, demonstrating that the double arginine residues at the p-5/p-4 positions upstream of the core PBM have an important role in the interaction with MAGI-1, in agreement with previous studies (Thomas et al., 2008; Zhang et al., 2007).

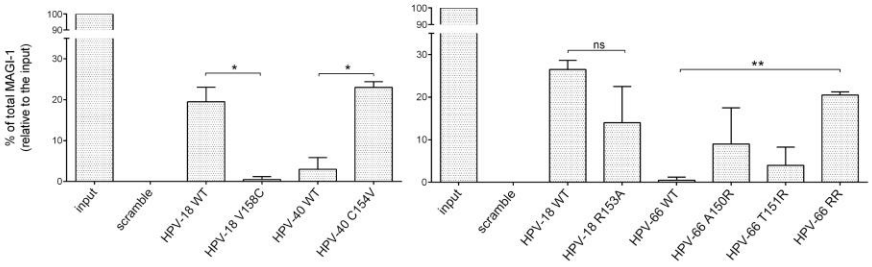
a.

	p-5 p-4 p-2 p-0		p-5 p-4 p-2 p-0
Scramble	R R L Q R T V E Q R	Scramble	R R L Q R T V E Q R
HPV-18 WT	R L Q R R R E T Q V	HPV-18 WT	R L Q R R R E T Q V
HPV-18 V158C	R L Q R R R E T Q C	HPV-18 R153A	R L Q R A R E T Q V
HPV-40 WT	E K G Q R S E T L C	HPV-66 WT	T S R Q A T E S T V
HPV-40 C154V	E K G Q R S E T L V	HPV-66 A150R	T S R Q R T E S T V
		HPV-66 T151R	T S R Q A R E S T V
		HPV-66 A150R/T151R	T S R Q R R E S T V

b.



c.



d.

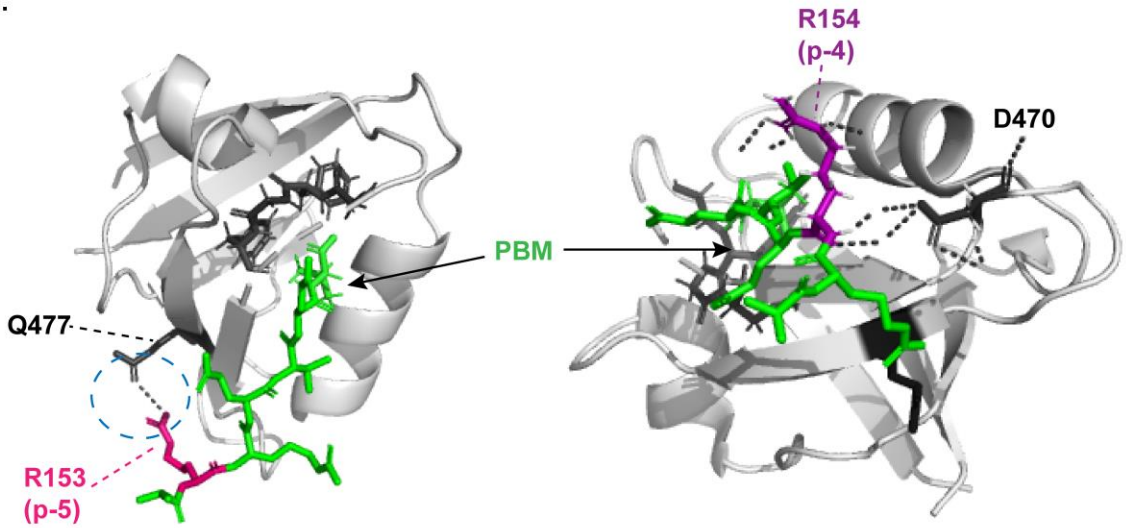


Figure 13. HPV E6 PBM binding to MAGI-1 analysed by peptide pulldown assay. (a) The panel shows the wild-type and mutant sequences of the biotinylated peptides corresponding to the PBMs of HPV-18, HPV-40 and HPV-66 E6 proteins. The 'scramble' peptide, corresponding to the scrambled sequence of 18PBM was used as a negative control. The wild-type amino acids are underlined and the mutations are shown in red. (b) The peptides on (a) were conjugated to magnetic streptavidin beads and used in pull-down assays with the total cell extract from HEK293 cells transfected with Flag-MAGI-1. Bound Flag-MAGI-1 was analyzed by Western blotting with anti-FLAG antibody. (c) Protein band intensities relative to the experiment shown in (b) were determined using Image J and GraphPad Prism 7 programs. Levels of the indicated proteins were normalized to the relative 100% input. The histograms show the quantitation of the mean and the standard deviations from at least three independent assays. Student's t-test was used for calculating the significance (*p<0.05; **p<0.01; ns=not significant). (d) The structure of MAGI-1 PDZ1 domain and HPV-18 PBM was modelled using PyMol software (pdb:2I04). The carboxyl group of the 18E6 peptide is well anchored to the GFGF motif on MAGI-1 PDZ, which suggest the importance of the p-0 valine residue in PDZ binding, and the critical role of the C/V substitution. Moreover, it has been previously shown most PDZ-PBM interaction complexes need the 4 canonical residues for interaction, but at least six residues participate in binding to PDZ and depending on the type of interaction, up to 9 residues are important for E6 binding. Indeed, a point mutation in p-4 of HPV-18 abolish E6-mediated degradation of hDlg1 and MAGI-1. Furthermore, the residue on p-5 is also important for MAGI-1 binding. This residue's side chain interacts with the Q477 polar group in the PDZ. This is not observed with hDlg1 PDZ2/PDZ3, because there is a hydrophobic phenylalanine residue instead. This is why p-5 is not critical for hDlg1 binding, but it is for MAGI-1.

The results with hDlg1 are shown in Figure 14. Again, the critical role of the terminal C/V residue between HPV-18 and -40 was confirmed, albeit this is not as critical as it is for MAGI-1. This is also in agreement with previous studies (Thomas et al., 2016). In the case of the upstream residues important for PKA phosphorylation, the HPV-18 R153A mutant decreases interaction

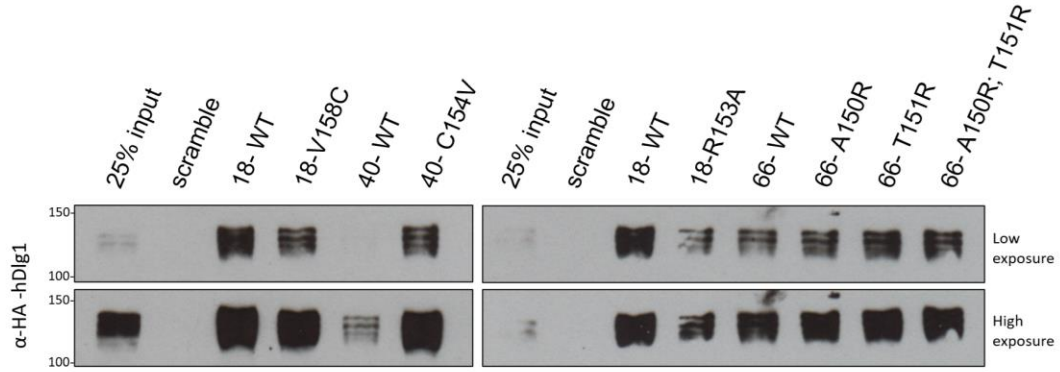
with hDlg1. Likewise, for HPV-66, all the arginine substitutions result in a mild increase in the ability of the mutant peptides to bind hDlg1.

a.

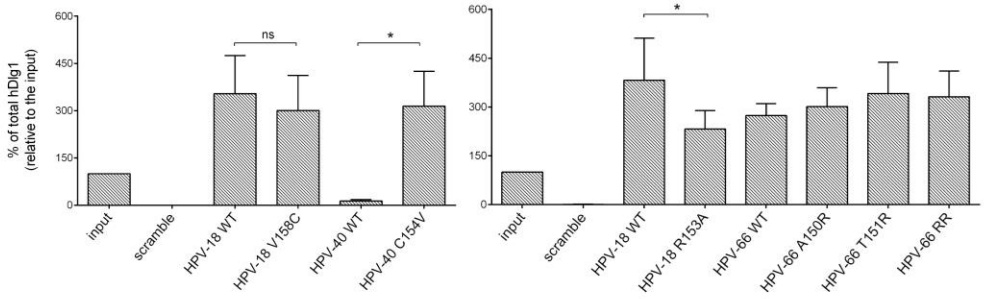
	p-5 p-4 p-2 p-0
Scramble	R R L Q R T V E Q R
HPV-18 WT	R L Q R R R E T Q V
HPV-18 V158C	R L Q R R R E T Q C
HPV-40 WT	E K G Q R S E T L C
HPV-40 C154V	E K G Q R S E T L V

	p-5 p-4 p-2 p-0
Scramble	R R L Q R T V E Q R
HPV-18 WT	R L Q R R R E T Q V
HPV-18 R153A	R L Q R A R E T Q V
HPV-66 WT	T S R Q A T E S T V
HPV-66 A150R	T S R Q R T E S T V
HPV-66 T151R	T S R Q A R E S T V
HPV-66 A150R/T151R	T S R Q R R E S T V

b.



c.



d.

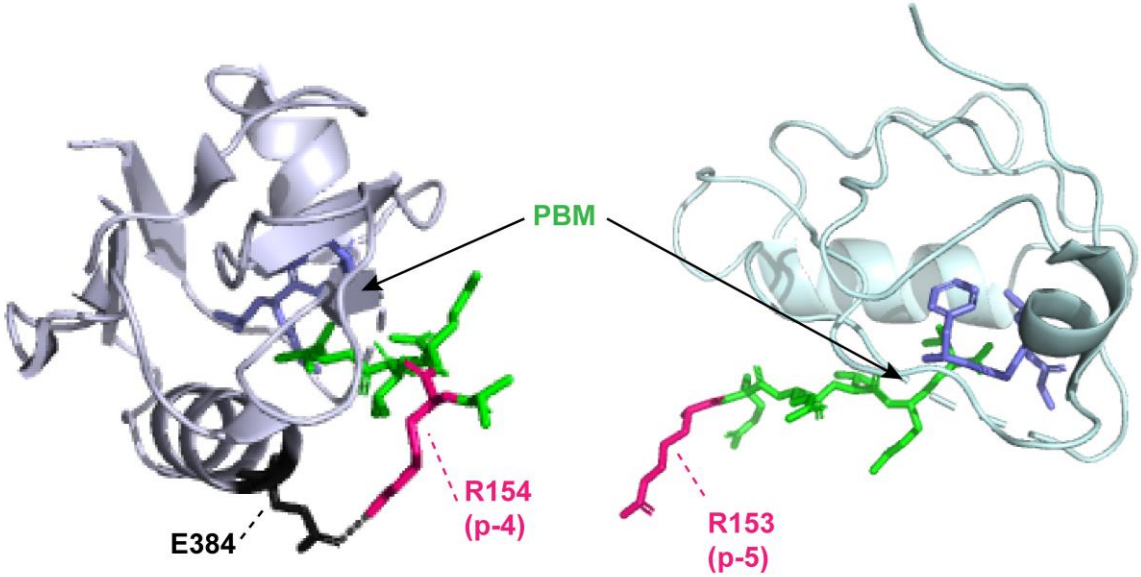


Figure 14. HPV E6 PBM binding to hDlg1 analysed by peptide pulldown assay. (a) The panel shows the wild-type and mutant sequences of the biotinylated peptides corresponding to the PBMs of HPV-18, HPV-40 and HPV-66 E6 proteins. The 'scramble' peptide, corresponding to the scrambled sequence of 18PBM was used as a negative control. The wild-type amino acids are underlined and the mutations are shown in red. (b) The peptides on (a) were conjugated to magnetic streptavidin beads and used in pull-down assays with the total cell extract from HEK293 cells transfected with HA-hDlg1. Bound hDlg1 was analyzed by Western blotting with anti-HA antibody. (c) Protein band intensities relative to the experiment shown in (b) were determined using Image J and GraphPad Prism 7 programs. Levels of the indicated proteins were normalized to the relative 100% Input. The histograms show the quantitation of the mean and the standard deviations from at least three independent assays. Student's t-test was used for calculating the significance (*$p<0.05$; ns=not significant) (d) The structure of DLG1-PDZ2 domain and HPV-18 PBM was modelled using PyMol software (pdb:2I0L). Furthermore, the arginine residue on p-5 is not critical for hDlg1 binding, and we observed in the structure that indeed, the residue has an outer expose position, not forming hydrogen bonds or close contacts with residues on the PDZ domain itself. Moreover, the arginine residue in p-4 position, which makes a hydrogen bond with the E384 residue on the DPZ, seems to have a greater effect in binding.

Finally, we also analysed association with hScrib, and the results obtained are shown in Figure 15. Previous results had shown that the capacity to interact with Scribble was very closely linked with cancer-causing potential (Thomas et al., 2016), and this is supported by these results, with only HPV-18 E6 showing a significant level of interaction with hScrib. In addition, the HPV-18 E6 V158C substitution destroys the ability of HPV-18 E6 to bind hScrib, as expected, whilst the C154V substitution in HPV-40 failed to confer interaction, indicating that a carboxy terminal cysteine is incompatible with hScrib recognition, but that a simple replacement of the cysteine with a valine alone is also not sufficient to confer interaction with hScrib. In agreement with this, the arginine introduction at the p-5 position, which has no effect on PKA phosphorylation of HPV-66, also failed to confer interaction with hScrib; whilst the arginine residues introduced in p-4 and p-5/p-4 conferred a significant degree of interaction with hScrib and also allowed

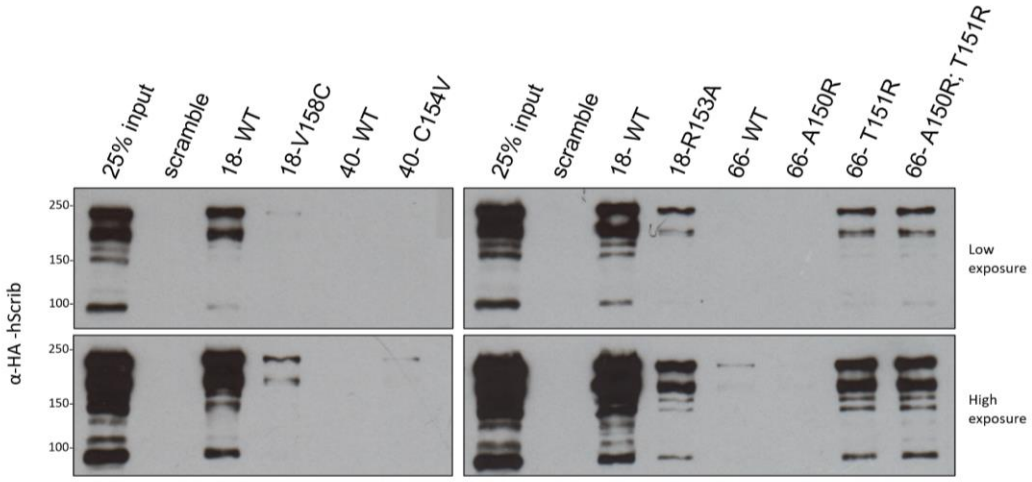
phosphorylation by PKA. Taken together, these results demonstrate that multiple residues within the E6 PBM play critical roles in hScrib recognition and, furthermore, these same residues play a critical role in conferring optimal levels of phosphorylation.

a.

	p-5 p-4 p-2 p-0
Scramble	R R L Q R T V E Q R
HPV-18 WT	R L Q R R R E T Q V
HPV-18 V158C	R L Q R R R E T Q C
HPV-40 WT	E K G Q R S E T L C
HPV-40 C154V	E K G Q R S E T L V

	p-5 p-4 p-2 p-0
Scramble	R R L Q R T V E Q R
HPV-18 WT	R L Q R R R E T Q V
HPV-18 R153A	R L Q R A R E T Q V
HPV-66 WT	T S R Q A T E S T V
HPV-66 A150R	T S R Q R T E S T V
HPV-66 T151R	T S R Q A R E S T V
HPV-66 A150R/T151R	T S R Q R R E S T V

b.



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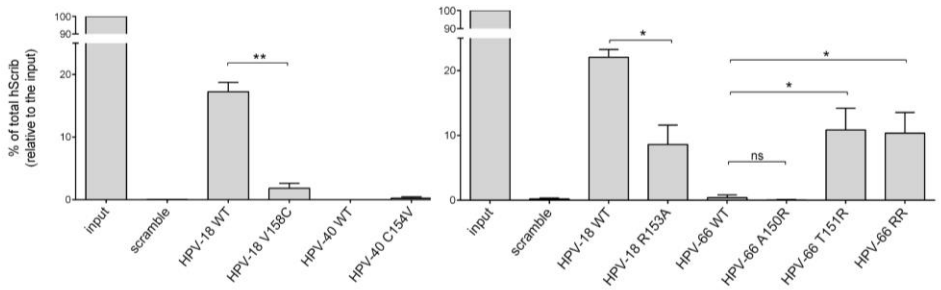

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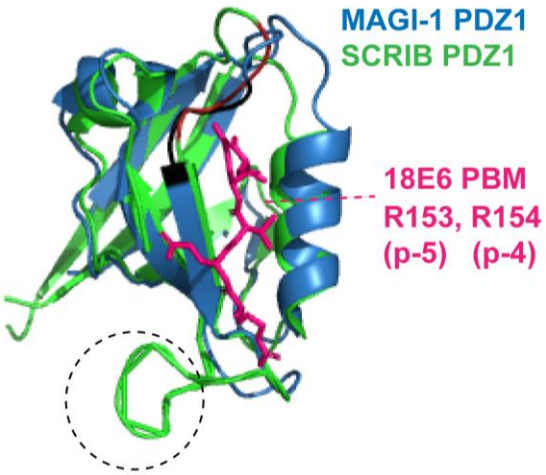


Figure 15. HPV E6 PBM binding to hScrib analysed by peptide pulldown assay. (a) The panel shows the wild-type and mutant sequences of the biotinylated peptides corresponding to the PBMs of HPV-18, HPV-40 and HPV-66 E6 proteins. The 'scramble' peptide, corresponding to the scrambled sequence of 18PBM was used as a negative control. The wild-type amino acids are underlined and the mutations are shown in red. (b) The peptides on (a) were conjugated to magnetic streptavidin beads and used in pull-down assays with the total cell extract from HEK293 cells transfected with HA-Scrib protein. Bound hScrib was analyzed by Western blotting with anti-HA antibody. (c) Protein band intensities relative to the experiment shown in (b) were determined using Image J and GraphPad Prism 7 programs. Levels of the indicated proteins were normalized to the relative 100% input. The histograms show the quantitation of the mean and the standard deviations from at least three independent assays. Student's t-test was used for calculating the significance (*$p<0.05$; **$p<0.01$; ns=not significant). (d) The structure of MAGI-1 PDZ1 in complex with HPV-18 PBM (pdb:2I04) was aligned with the hScrib PDZ1 domain (pdb:1X5Q) and modelled using PyMol software. Indeed, hScrib structure (green) has an extra loop (dashed circle), not present in MAGI1 or hDlg1, which may need extra residues upstream of the PBM to stabilize the interaction.

Part 2: HPV-16 E6 phosphorylated by PKC and CK1 kinases modulates E6 distribution and PDZ protein interactions.

Although the previous part is focused on the phosphoregulation of HPV-18 PBM, HPV-16 contains specific phosphorylatable residues upstream of the PBM which might represent important targets for phosphoregulation. Furthermore, although the global prevalence of high-risk (HR) HPV types and the incidence of cervical cancer vary with the continent and the country, HPV-16 is still the most frequently detected high-risk type and is the predominant type causing invasive cervical cancer worldwide (~ 60%) (Bouvard et al., 2009; Bruni et al., 2019; Bruni et al., 2010; Ciapponi et al., 2011).

Although, HPV-18 and HPV-16 have the most promiscuous E6 proteins, they belong to different species groups within the high-risk clade of the alpha HPV tree. Indeed, they present a significant variability in their non-structured carboxy-terminal sequences (Figure 2). As previously mentioned, the PDZ proteins can recognize up to the last nine residues at the carboxyl terminus of the E6 PBM (Charbonnier et al., 2011; Fournane et al., 2011; Nominé et al., 2003; Thomas et al., 2008; Tonikian et al., 2008; Zhang et al., 2007), which affect the spectrum of PDZ binding partners of E6 and this correlates with the cancer risk classification (groups 1, 2A, 2B and 3) (Thomas et al., 2005, 2016).

Besides mediating interaction of E6 with members of the polarity complexes, the PDZ-PBM interaction results in stabilization of E6, since it has been shown that the highly unstructured PBM acquires a defined structure once in complex with a specific PDZ domain (Nominé et al., 2003, 2006; Tonikian et al., 2008), suggesting that this association might not only help E6 degrade its targets, but ensure that sufficient levels of E6 are present in the cells (Nakagawa & Huibregtse, 2000). An example of this is the pool of E6 that is stabilized by hScrib and which may not be involved in p53 degradation. This is supported by previous observations showing that the levels of expression of an E6 mutant defective in PDZ recognition were still sufficient to degrade p53 (Pim et al., 1994). All this implies that the PDZ-PBM interaction may have additional functions to inducing PDZ protein degradation, and that phosphorylation of the E6 PBM might not only affect association with the PDZ domain but might still play a role in correct functioning of E6 or its target proteins.

Furthermore, in Part 2, we show that the E6 promiscuity, conferred by core and upstream residues of the PBM, appears to have co-evolved with a consensus phosphorylation site embedded within the PBM, and this adds to the functional diversity of the E6 PBM.

The PDZ-binding motif is one of the most common protein–protein interaction motifs and phosphorylation at position −2 of the class I motif is widely reported to modulate this type of interaction (Lee & Zheng, 2010b), as observed with HPV E6. Although it has been previously

shown that residues upstream of the PBM do not significantly affect its affinity for any given PDZ domain, it could be speculated that phosphorylation of these upstream residues might provide an additional mechanism for modulating the interaction with PDZ targets. Indeed, several other positions in the PBM have been reported to be phosphorylated (Chen et al., 2008; Chung et al., 2000; Matsuda et al., 1999; von Nandelstadh et al., 2009). The serine residue at p-4 in the PBM of the AMPA receptor GluR2 subunit is phosphorylated by PKC *in vitro* and *in vivo*; and the serine residue at p-5 of the LRPP4 carboxy-terminal cytoplasmic region is phosphorylated by CamKII (Chung et al., 2000; Matsuda et al., 1999; Tian et al., 2006). Furthermore, the ZO-1 PDZ2 domain interacts with the PBM of Connexin 43 (Cx43) and this interaction is regulated by AKT and PKC phosphorylation of serine residues at p-9 and p-10 in the Cx43 PBM (Madoz-Gúrpide et al., 2007; Park et al., 2007; Solan et al., 2003, 2007; Solan & Lampe, 2009).

Additionally, studies with peptides arrays, able to simultaneously screen several different peptides on cellulose surfaces, better recognized as SPOT synthesis techniques, have evaluated 3 arrays of 100 PDZ-binding sequences and all their possible phosphorylated variants were analyzed against 3 PDZ domains. The interaction of 344 peptides for AF-6 PDZ, 319 peptides for ERBIN PDZ, and 355 peptides for the α-1-syntrophin PDZ domains showed that phosphorylation of the PDZ-binding motif at p-2 and at p-1 significantly inhibited PDZ-mediated interactions; while phosphorylation at p-4, p-7, and p-8 only slightly affected the interactions, depending on the PDZ domain; and phosphorylation at p-3, p-5, p-6, p-9, or p-10 had little or no influence on the interactions (Boisguerin et al., 2007).

While many studies have reported that phosphorylation at the carboxy-terminus of PDZ-binding proteins negatively regulates the PDZ interactions, others have shown that phosphorylation can also promote PDZ interactions (Adey et al., 2000; von Nandelstadh et al., 2009). Phosphorylation by PKA and PKC of the p-6 in the PBM of the NR2C subunit of NMDAR did not change its binding to the PSD-95 PDZ3 (Chen et al., 2006), but the phosphomimic mutations accelerated the channel kinetics, altering the function of the NMDA receptor channels, suggesting that phosphorylation of the PBM can still play an additional role in the function of its target protein.

In this part we report additional phosphorylation sites located in the upstream sequence of the core PBM of HPV-16 E6. Interestingly, the serine residues evaluated comprise an important structural part of E6, but their phosphorylation did not seem to affect p53 degradation or the interaction with PDZ proteins hDlg1 and hScrib; however, they did affect E6's cellular distribution. Furthermore, phosphorylation events closer to the PBM seemed to affect E6's interactions with other, less-studied, PDZ proteins, like β2-Syntrophin and TIP-1.

Furthermore, using in vitro assays we were able to identify two possible kinases, PKC and CK1, responsible for phosphorylating these sites.

HPV-16 E6 phosphorylation upstream of the PBM positively regulates interaction with certain PDZ proteins.

Initially, we assessed the importance of the T152 residue on position -6 of the 16PBM by proteomic analysis (Appendix I, Figure 16). We generated biotinylated-peptides with the last 10 carboxy-terminal residues of HPV-16 E6. The wild-type peptide, the scramble peptide, the phospho-peptide of position -2 (pT-2), which has previously shown to abolish PDZ interaction, and the phospho-peptide of position -6 (pT-6) were used in pull-down assays with HACAT cell extracts (Figure 16d). The quality of the pull-down was confirmed by western blot using hDlg1 antibody (Figure 16c). The pulled-down proteins were then analysed by mass spectrometry analysis and the results are shown in Figure 16b and Appendix I. As expected, the wild-type binds its target proteins; the 16E6 pT-2 decreases or abolishes most PDZ interactions, and the 16E6 pT-6 peptide does not seem to affect most common targets but show specific differences in binding depending on the PDZ proteins. Interestingly, the 16E6 pT-6 peptide increases binding to TIP-1 (Tax1 Binding Protein 3) and SNTB2 (Syntrophin Beta 2 or β2-Syntrophin), when compared with the wild-type. Furthermore, the 16E6 pT-6 and 16E6 pT-2 peptides decrease similarly biding to ZO-2 (Zona occludens 2) protein, also known as Tight Junction Protein 2 (Figure 16b).

We corroborated the proteomic analysis results performing pull-down assay using the biotinylated peptides with total cells extracts from HEK293 cells transiently expressing TIP-1 and SNTB2 proteins (Figure 16e and 16g). As shown, the 16E6 pT-6 peptide increases the interaction with both proteins (blue bars in Figures 16f and 16h). We probed for endogenous Scribble as a control (gray bars in Figures 16f and 16h). As expected, the wild-type strongly binds hScrib, 16E6 pT-2 peptide abrogated binding to hScrib, and the 16E6 pT-6 peptide does not affect critically the binding to hScrib, confirming that the results observed are not due to the differences in the concentration or the degradation of the peptide.

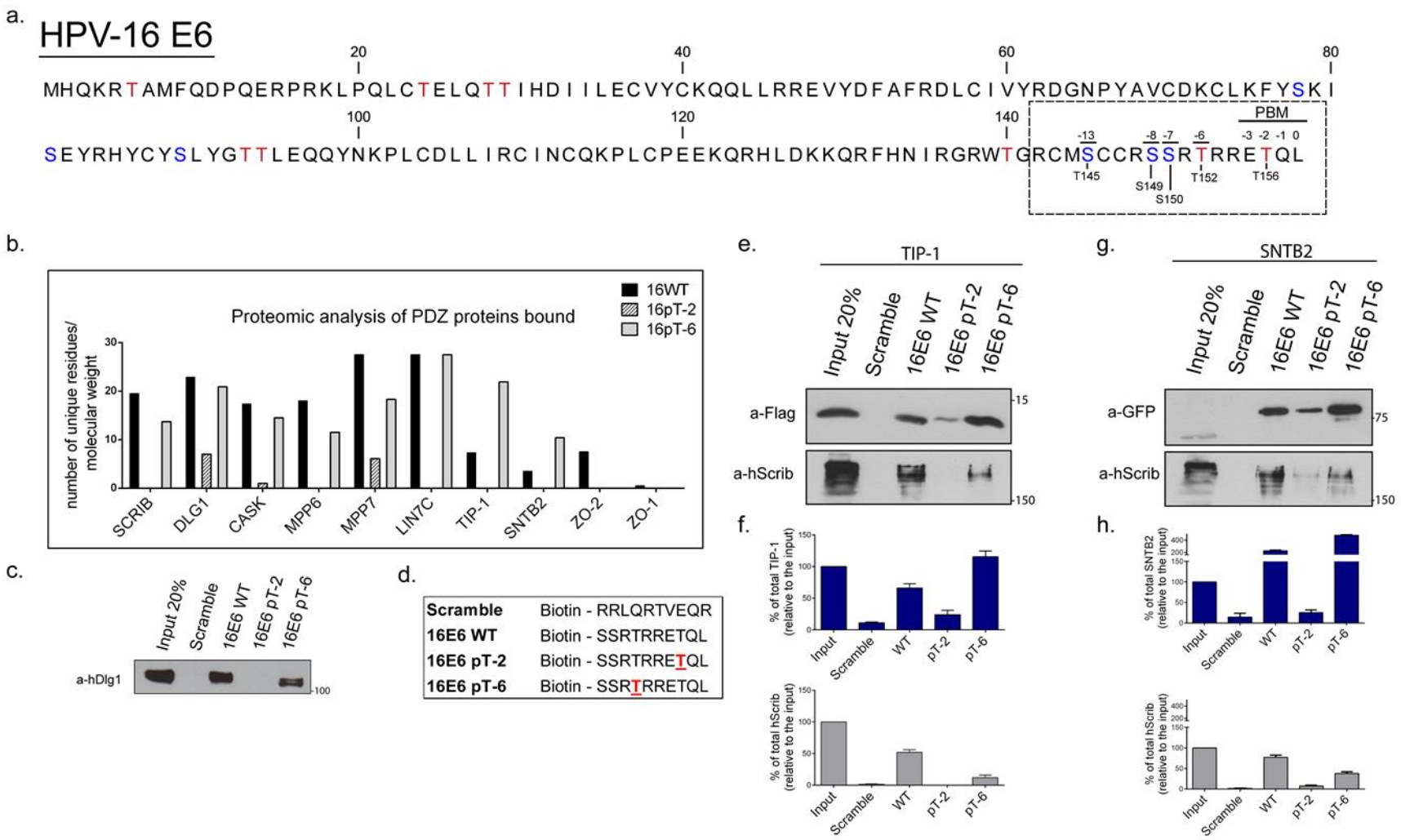

a.
HPV-16 E6
MHQKRTAMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVYDFAFRDLCIVYRDGNPYAVCDKCLKFYSKI
SEYRHYCYSLYGTTLEQQYNKPLCDLLIRCINCQKPLCPEEKQRHLDKKQRFHNIRGRWTGRCMSCCRSSRTRRETQL
PBM
T145
S149
S150
T152
T156
b.
Proteomic analysis of PDZ proteins bound
16WT
16pT-2
16pT-6
number of unique residues/ molecular weight
SCRIB
DLG1
CASK
MPP6
MPP7
LIN7C
TIP-1
SNTB2
ZO-2
ZO-1
c.
Input 20%
Scramble
16E6 WT
16E6 pT-2
16E6 pT-6
a-hDlg1
d.
Scramble Biotin - RRLQRTVEQR
16E6 WT Biotin - SSRTRRETQL
16E6 pT-2 Biotin - SSRTRRETQL
16E6 pT-6 Biotin - SSRTRRETQL
e.
TIP-1
a-Flag
a-hScrib
f.
% of total TIP-1 (relative to the input)
% of total hScrib (relative to the input)
g.
SNTB2
a-GFP
a-hScrib
h.
% of total SNTB2 (relative to the input)
% of total hScrib (relative to the input)

Figure 16.- The effect of phosphorylation on T152 in PDZ proteins binding. (a) Threonine and serine residues are shown in red and blue colors, respectively. The dash lined box includes the residues evaluated and the PBM, and the positions in the sequence and the number of the residue are shown. (b) Magnetic streptavidin beads coated with the biotin-PBMs from HPV-16 and the phosphorylated mutants were used in pull-down assays with the total cell extract from asynchronous HaCaT cells and the bound proteins were analyzed by mass spectrometry (Appendix I). The graph shows the unique peptides for each protein normalized against the molecular weight (Y axis), and the name of the target proteins bound (X axis). (c) The 5% of the eluted bound proteins from (b) was analyzed by Western blotting with anti-hDlg1 as a control. (d) The sequences of the peptides used are shown. The phosphorylated residues are marked in red. (e and g) Magnetic streptavidin beads coated with the biotin-PBMs from HPV-16 and the phosphorylated mutants were used in pull-down assays with the total cell extract from HEK293 cells transfected with FLAG-TIP-1 (e) or GFP-SNTB2 (g), and analyzed by Western blotting with anti-GFP, anti-FLAG and anti-hScrib as a control. (f and h) The graphs represent the results from two independent western blot experiments with the respective standard deviations, where the blue bars show the percentage of protein bound relative to the input for TIP-1(f – upper graph) and SNTB2 (h- upper graph), and, the grey bars, for hScrib as a binding control (f and h, bottom panels).

To further characterize the effect of phosphorylation of T152 in position p-6 of HPV-16E6, we used purified HPV-E6 glutathione S-transferase (GST) fusion proteins and generated several mutants that mimic phosphorylation in position p-6 and the corresponding alanine mutant in the context of the full-length E6 protein (Mutant sequences are shown in Figure 18c). Figure 17 shows the results of the pull-down assays using GST proteins and total cell extracts from HEK293 cells transfected with Flag-tagged TIP-1 and GFP-tagged SNTB2. As shown in Figure 17a, the wild-type E6 binds TIP-1. Surprisingly, the T156A mutant, unable to be phosphorylated on Tp-2 of the PBM, binds strongly to TIP1 proteins. This correlates with the fact that Tp-2 does not completely abrogates binding to TIP-1 and that T152A mutant decreases binding more than the T156A mutant, suggesting the threonine residue on this

position is critical for binding TIP-1. Furthermore, the T152D mutant slightly increases binding of E6 to TIP1 in comparison with the wild-type E6. This suggest that T152 is more important for TIP-1 binding than T156 on the PBM (Figure 17a), indicating that TIP-1 recognition is quite distinct from that of the other E6 PDZ domain containing substrates.

In the case of SNTB2, the alanine mutant T152A affects binding and the phospho-mimic mutant T152D slightly increases it, in comparison to the wild-type, in a manner similar to what was observed for TIP-1. However, the T156A mutant of the E6 PBM dramatically inhibited binding to SNTB2, indicating that SNTB2 is recognized like most other PDZ targets of E6, with the integrity of T156 being critical. In the case of both TIP-1 and SNTB2, the T152E substitution does not seem to behave as a proper phospho-mimic (Figure 17b), indicating subtle differences as to whether an aspartic or glutamic acid residue is chosen to represent a phospho mimic.

We wanted to determine if the phospho-mimic mutants used above were indeed decreasing binding to ZO-1 as was observed in the proteomic analysis (Figure 16b). We did the GST pull-down assays with cells extracts from HEK293 cells transiently expressing HA-tagged ZO-1 protein. As expected, we observed that both mutants decrease binding to a level similar to the ΔPBM mutant., in a manner similar to that seen for hScrib (Figure 17d). These results reinforce the concept that residues upstream of the PBM have important and specific effects on diverse PDZ target proteins and in the case of two substrates, SNTB2 and TIP-1 phosphorylation at T152 increases PDZ recognition.

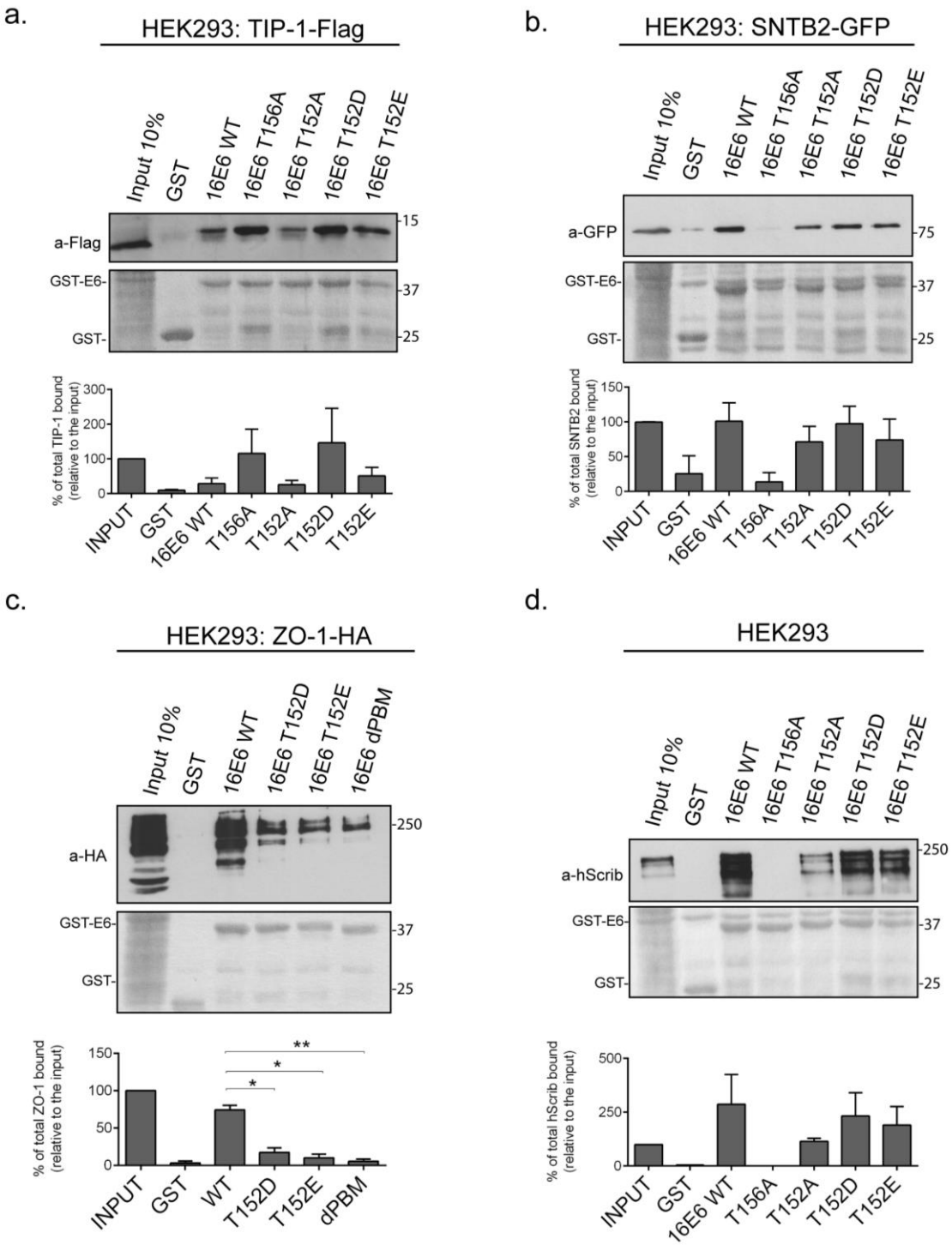


Figure 17.- HPV-16 E6 T152 residue affects binding to PDZ proteins. HPV-16 E6 GST fusion proteins were generated. Phospho-mimic and alanine mutations were introduced in the position p-6 of E6. The HPV-16

GST E6 and the corresponding mutants were used in pull-down assays with the total cell extract from HEK293 cells transfected with FLAG-TIP1 (a), GFP-SNTB2 (b) and HA-ZO1 (c) and anti-hScrib. All pull-down experiments were analyzed by Western blotting with anti-GFP, anti-FLAG, anti-HA and anti-hScrib antibodies. The lower panels in each figure show the GST loading in Ponceau S staining. The bar graphs show the mean and standard deviation of the percentage of the total of protein bound, relative to the input, from at least three independent experiments. Student's t-test was calculated (*$p<0.05$; **$p<0.01$), but significance was only observed for ZO-1.

HPV-16 E6 contains unique consensus phosphorylation motifs in its carboxy-terminal sequence.

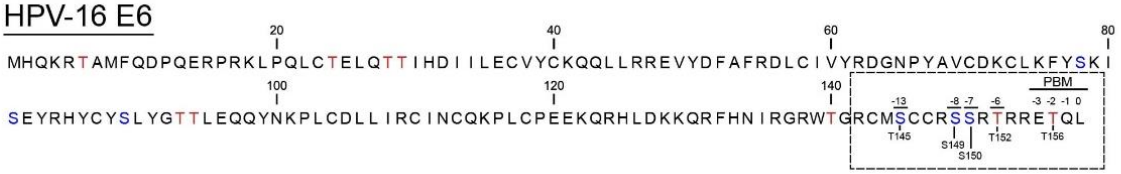
The above studies show that T156 and T152 can play a major role in substrate recognition and the function of the E6 PBM, depending on the level of phosphorylation and the identity of the specific PDZ domain containing substrate. Whilst much information is known about the identity of kinases that phosphorylate T156, there is no information on which kinases might phosphorylate T152. Therefore, we used the NetPhos 3.1 server to *in silico* predict serine and threonine phosphorylation sites on HPV-16 (Blom et al., 1999, 2004). The prediction score of the output has a 0.00 to 1.00 scale, above 0.500, indicates a positive prediction. We selected only the results with scores higher than 0.250. From all the kinases evaluated, we found that PKC has a high positive score of 0.739 on T152 and that the consensus phosphorylation sequence is present repeatedly in HPV-16 E6 (Figure 18a and 18b).

Furthermore, we observed a triplet of serine residues (S145, S159, S150) on the carboxy-terminal sequence, each one with the respective arginine residues upstream, which were also identified as possible PKC phospho-acceptor sites (Figure 18a). These three serine residues are specific for HPV-16 E6 and are not present in other high-risk or low-risk types analysed (Figure 19b) and not in any of the other high-risk types of the alpha phylogenetic tree (Figure 2).

Despite several other potential phospho-acceptor sites that were identified (Figure 18a), we focused on the serine triplet and T152 as shown in Figure 18c.

a.

HPV-16 E6



	78S	81S	89S	145S	149S	150S
unsp	0	0.985	0	0.958	0	0.987
CaM-II	0.451	0.435	0.449	0.459	0.454	0.425
cdc2	0.414	0.528	0.413	0.484	0.425	0.49
CKI	0.364	0.365	0.435	**0.371**	**0.372**	**0.383**
CKII	0.368	0.4	0.313	0	0.305	0
cdk5	0	0	0	0.336	0.369	0
DNAPK	0.338	0.342	0.423	0.359	0.345	0.398
GSK3	0.434	0.419	0.456	0.469	0.455	0.444
PKC	0	0	0.743	**0.445**	**0.617**	**0.471**
PKA	0.386	0	0.598	0.365	0	0.735
PKB	0	0	0	0	0	0
PKG	0.374	0.373	0	0.336	0.352	0.329
p38MAPK	0	0.349	0	0.403	0.352	0
RSK	0	0.325	0	0.492	0	0.368

	6T	24T	28T	29T	93T	94T	140T	152T	156T
unsp	0	0	0	0	0	0	0.87	0.975	0.646
ATM	0	0	0	0	0	0	0	0	0.448
CaM-II	0.457	0.44	0.415	0.448	0.426	0.428	0.458	0	0.47
cdc2	0.425	0.401	0.368	0.398	0.468	0.423	0.343	0	0.374
CKI	0.361	0.357	0.36	0.387	0.359	0.368	0.36	**0.361**	**0.371**
CKII	0.316	0.371	0.478	0.375	0.538	0.347	0	0	0.295
cdk5	0	0	0	0	0	0	0	0	0.24
DNAPK	0.339	0.337	0.337	0.339	0.339	0.353	0.342	0.339	0.513
GSK3	0.445	0.424	0.43	0.421	0.472	0.431	0.45	0.439	0.459
PKC	0.498	0.552	0	0	0.417	0	0.799	**0.739**	**0.243**
PKA	0	0	0	0	0	0	0.39	0	0.414
PKB	0	0	0	0	0	0	0	0	0.647
PKG	0.604	0	0	0	0.377	0.304	0	0	0.518
p38MAPK	0.307	0.329	0.332		0.301	0	0	0	0.281
RSK	0	0	0	0	0	0	0	0	0.552

b.

PKC → R – R – **S/T**
R – X – **S/T**

CK1 → pS/D/E – X – X - **S/T**

c.

16 E6 WT		MSCCRSSRTRRETQL
16 E6 ΔPBM		MSCCRSSRTRR- - - -
16 E6 T156A		MSCCRSSRTRREAQL
16 E6 T152A		MSCCRSSRARRETQL
16 E6 T152D		MSCCRSSRDRRETQL
16 E6 T152E		MSCCRSSRERRETQL
16 E6 S145A		MACCRSSRTRRETQL
16 E6 S149A; S150A	(2A)	MSCCRAARTRRETQL
16 E6 S145A; S149A; S150A	(3A)	MACCRAARTRRETQL
16 E6 S149D; S150D	(2D)	MSCCRDDRTRRETQL
16 E6 S145D; S149D; S150D	(3D)	MDCCRDDRTRRETQL

Figure 18.- HPV-16 E6 amino acid sequence and predicted phosphorylation sites upstream of the PBM. (a) HPV-16 E6 amino acid sequence. Threonine and serine residues are shown red and blue colors, respectively. The dash lined box englobe the residues evaluated and the PBM, the positions in the sequence and the number of the residue. (Left) The blue heat map shows the scores generated by the NetPhos3.1 software for all serine residues (horizontal), and the kinases evaluated. The increase in blue color intensity represent higher scores and the bolded numbers, represent the scores of the residues assessed. (Right) The red heat map shows the scores generated by the NetPhos3.1 software for all threonine residues (horizontal), and the kinases evaluated. The increase in red color intensity represent higher scores and the bolded numbers, represent the scores of the residues assessed. (b) PKC and CK1 phospho-consensus sequences. (c) The sequences of the HPV-16 E6 GST fusion proteins

generated are shown. Green and red colors represent alanine and aspartate substitutions, respectively.

HPV-16 E6 is phosphorylated by PKC and CK1 kinases.

We performed a series of in vitro kinase assays using purified kinase PKC and purified HPV-E6 glutathione S-transferase (GST) fusion proteins as substrates in the presence of radiolabeled ATP. We observed that HPV-16 E6 is highly phosphorylated by PKC (Figure 19a). Interestingly, phosphorylation of the ΔPBM and T152A mutants by PKC kinase did not decrease significantly in comparison with the 16E6 wild-type phosphorylation, suggesting PKC phospho-acceptor site is located elsewhere.

Even though CK1 did not have a high phosphorylation score, we observed that it had a similar score in all possible phosphorylatable residues across the sequence, therefore, we decided to test if phosphorylation could occur even though the classical phospho-acceptor sequence was not present. In addition, it has been shown that CK1 is a priming kinase, which generates a complex chain of phosphorylation events, which can start earlier in the protein sequence. In the case of CK1, we used the purified kinase and purified HPV-E6 glutathione S-transferase (GST) fusion proteins as substrates in the presence of radiolabeled ATP. As shown in Figure 19b, 16E6 wild type is highly phosphorylated by CK1, the T152D did not greatly affect phosphorylation, and whilst it was somewhat reduced with the T152E mutation. CK1 phosphorylation was also reduced by the ΔPBM mutant, suggesting that phosphorylation by CK1 is indeed a complex event (Figure 20c).

Knowing that HPV-16 E6 is phosphorylated by PKC and CK1 but not primarily within the PBM or T152 residue, we compared the amino acid sequences of HPV-16 and representative high-risk and low-risk types and observed that even though many high-risk types have serine and threonine residues upstream of the PBM, only HPV-16 E6 has three possible phospho-acceptor sites upstream of position p-6, which includes a triple of serines, conformed by a S145 and a double serine motif S149;S150 (Figure 19c). Considering this, we firstly performed the in vitro phosphorylation assays using 16E6 wild-type protein, and the ΔPBM and T152A mutants as controls; and E6s from HPV types with single serine residues (HPV-31, -33, -35, -66 and -40), and a double serine/threonine motif (HPV-66) to investigate whether phosphorylation by PKC is a common event within the alpha group. As observed in Figure 19, none of the analysed types, except HPV-16 E6 were phosphorylated by PKC. Additionally, we observed that HPV-18 E6 is only very weakly phosphorylated by both PKC (Figure 19a)

and CK1 kinases (Figure 19b). These results indicate that of the high-risk types tested only HPV-16 E6 appears to be a good substrate for PKC and CK1 phosphorylation.

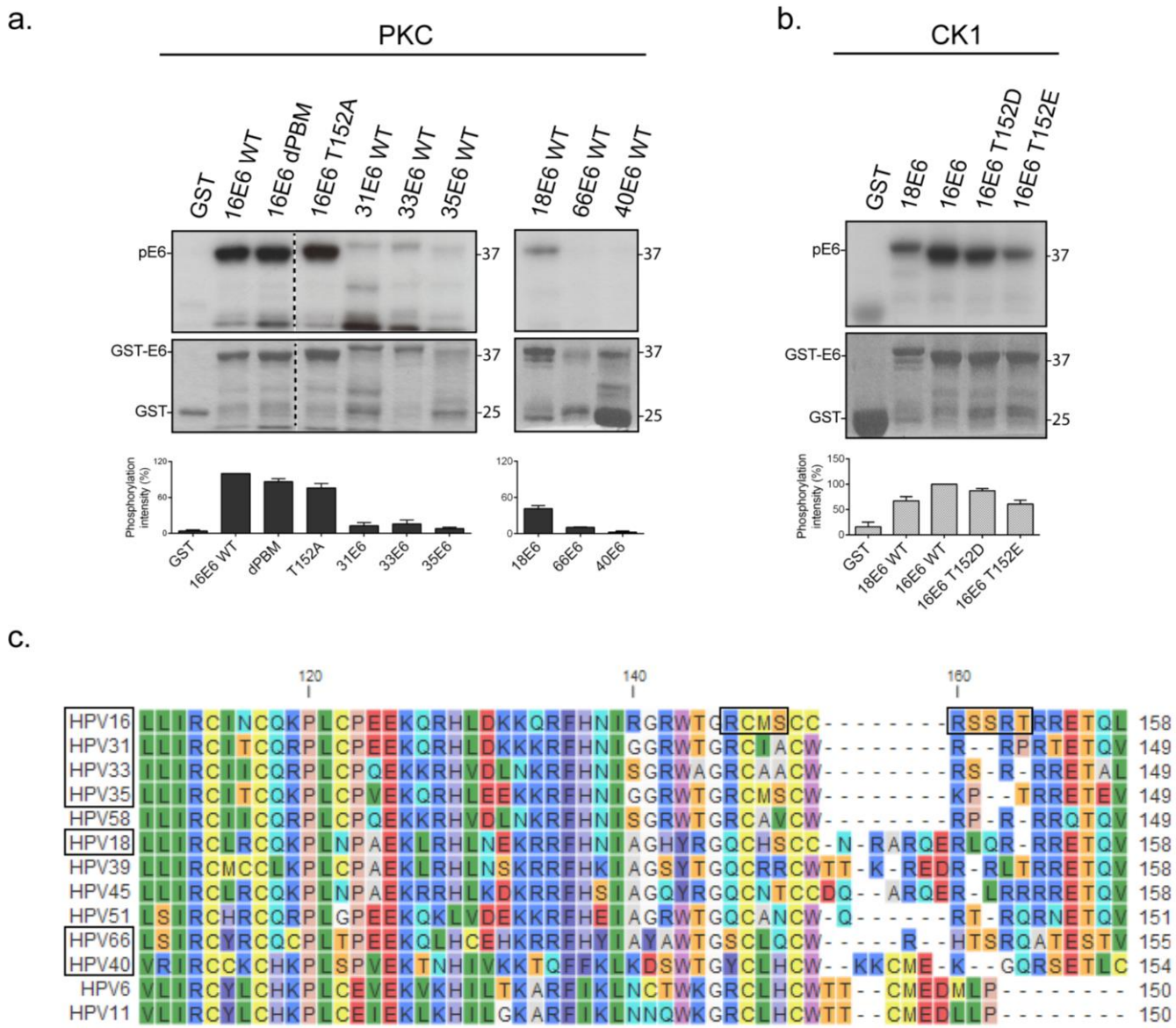


Figure 19.- PKC and CK1 kinases phosphorylate HPV-16 E6. (a) PKC *in vitro* phosphorylation of GST-E6 proteins with radiolabeled ɣ-^{32}P-ATP. The upper panels represent the phosphorylated proteins and the lower panel represent the GST loadings. The panels on (a, left) represent one radiograph, where the 18E6 sample was cut out since it was run on the same

gel, between 16E6 deltaPBM and 16E6 T152A samples, by mistake. The place where the gel was cut and edited is marked by a dashed line. The graph shows the percentage of phosphorylation intensity relative to the wild type 16E6. (b) CK1 *in vitro* phosphorylation of GST-16E6 proteins and its mutants with radiolabeled ɣ-^{32}P-ATP. The upper panels represent the phosphorylated proteins and the lower panel represent the GST loadings in Coomassie blue staining. The bar graphs show the mean and standard deviation of the phosphorylation intensity relative to the wild type 16E6, from two independent experiments.(c) Multiple amino acid sequence alignment of E6 sequences from selected high-risk and low-risk HPV types, showing the conservations of specific serine residues on HPV-16. The HPV types evaluated on (a) and the phospho-consensus sites for HPV-16, are marked by a black rectangle.

PKC and CK1 phosphorylate a triplet of serine residues in HPV-16 E6.

To further investigate whether the unique triplet of serine residues in HPV-16 E6 are phospho-acceptor sites for PKC and CK1 we generated the following alanine mutants: S145A; S149,S150A (2A) and S145A, S149A, S150A (3A) (Figure 18c); and performed a series of *in vitro* kinase assays using purified kinases PKC and CK1; and purified 16E6 glutathione S-transferase (GST) fusion proteins as substrate in the presence of radiolabeled ATP. As shown in Figure 20a and 20b, we observed a sequential decrease in phosphorylation by PKC as we introduced an alanine substitution. Indeed, the single and double alanine substitutions greatly decrease phosphorylation (Figure 20a), and when the three serine residues are substituted on the 3A mutant, the decrease in phosphorylation is even greater, suggesting that even though it seems that there are two different phospho-acceptor sites for PKC (RXXS an RSS), the presence of the three serine residues is critical for PKC phosphorylation (Figure 20b). Similar results were observed with CK1 phosphorylation (Figure 20c). Since a dramatic effect in phosphorylation was only observed when all three serines are mutated, we did not assess the effect of individually mutating the serines within the 2A mutant.

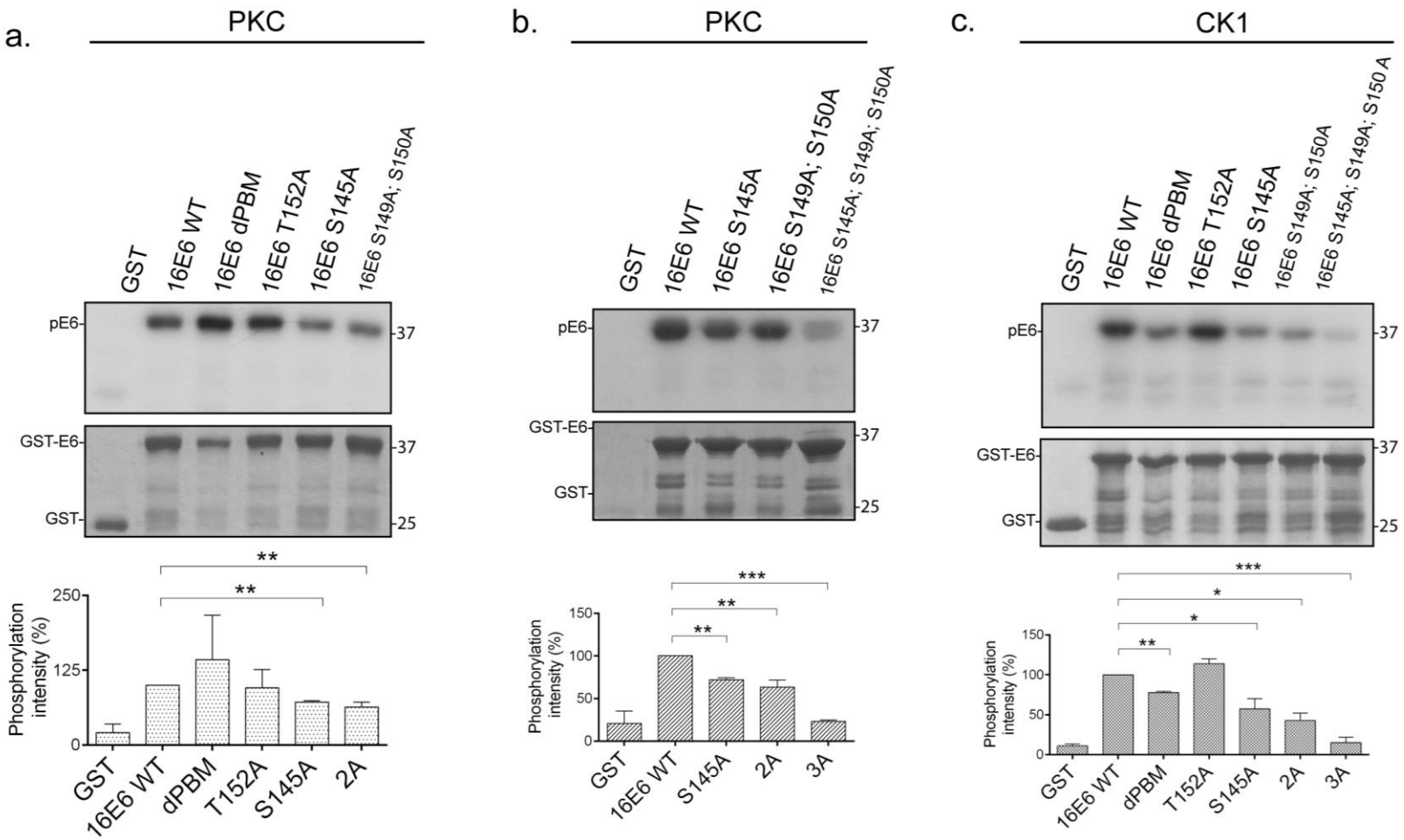

a.
PKC
GST
16E6 WT
16E6 dPBM
16E6 T152A
16E6 S145A
16E6 S149A; S150A
pE6
GST-E6
GST
37
25
Phosphorylation intensity (%)
250
125
0
**
**
GST
16E6 WT
dPBM
T152A
S145A
2A
b.
PKC
GST
16E6 WT
16E6 S145A
16E6 S149A; S150A
16E6 S145A; S149A; S150A
pE6
GST-E6
GST
37
25
Phosphorylation intensity (%)
150
100
50
0
***
**
**
GST
16E6 WT
S145A
2A
3A
c.
CK1
GST
16E6 WT
16E6 dPBM
16E6 T152A
16E6 S145A
16E6 S149A; S150A
16E6 S145A; S149A; S150A
pE6
GST-E6
GST
37
25
Phosphorylation intensity (%)
150
100
50
0
***
*
*
**
GST
16E6 WT
dPBM
T152A
S145A
2A
3A

Figure 20.- PKC and CK1 phosphorylate a triplet of serine residues in HPV-16 E6. (a and b) PKC *in vitro* phosphorylation of GST-16E6 wild type and its mutant proteins with radiolabeled ɣ-^{32}P-ATP. The upper panels represent the phosphorylated proteins and the lower panels represent the GST loadings in Coomassie blue staining. (c) CK1 *in vitro* phosphorylation of GST-16E6 wild type and its mutant proteins with radiolabeled ɣ-^{32}P-ATP. The upper panel represents the phosphorylated proteins and the lower panel represents the GST loadings in Coomassie blue staining. with the respective standard deviations. The student's t-test significance (* $p<0.05$) is shown. The bar graphs show the mean and standard deviation of the percentage of phosphorylation intensity relative to the wild type 16E6, from three independent experiments and the student's t-test significance (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

The double and triple phosphorylation event does not affect the functionality of in HPV-16 E6.

Although the serine residues are localized well away from the PBM, we wanted to test whether phosphorylation could affect binding capacity to the PDZ domains. Moreover, knowing the triple and double alanine and serine substitutions could potentially affect the structure of a E6, we wanted to assess the functionality of these mutants in the context of binding endogenous hScrib and hDlg1, and the less well studied targets, TIP-1 and SNTB2 proteins. The purified 16E6 glutathione S-transferase (GST) fusion wild-type and mutant proteins were incubated with total cell extracts from HaCaT cells and the bound proteins were detected by western blotting (Figure 21). As shown in Figure 21, the double and triple alanine and serine substitutions have a differential effect on binding to hScrib, hDlg1, SNTB2 and TIP-1. However the phosphomimic substitutions had a marked deleterious effect upon TIP-1 recognition and to a lesser degree also upon hScrib binding. In contrast the phosphor mimic substitutions had no effect on hDlg1 or SNTB2 recognition.

Furthermore, we assessed the function of the 16E6 mutants in the context of p53 degradation. We generated all the mutants in the HA-pCDNA3.1 vector for expression in mammalian cell lines. The H1299 cells were transfected with wild type p53, the empty vector as a control and each E6 mutant protein (Figure 22). As expected, the 16E6 wild-type protein degrades p53, and

all the mutants did similarly with some mild differences in the T152D and 2D mutants. This suggest that both phosphorylation on position p-6 and in the three upstream serine residues do not affect the main oncogenic function of E6 to degrade p53.

a.
Input 10%
GST
16E6 WT
16E6 dPBM
16E6 T156A
16E6 T152A
16E6 T152D
16E6 2A
16E6 3A
16E6 2D
16E6 3D
a-hScrib
GST-E6
GST
250
37
25
% of total hScrib bound
INPUT
GST
WT
dPBM
T156A
T152A
T152D
2A
3A
2D
3D
b.
a-hDlg1
100
% of total hDlg1 bound
c.
a-SNTB2
50
% of total SNTB2 bound
d.
a-TIP1
15
% of total TIP1 bound

Figure 21.- The double and triple phosphorylation events affect differentially PDZ proteins binding. The HPV-16 GST E6 wildtype and mutant proteins were used in pull-down assays with the total cell extract from asynchronous HaCat cells. All pull-down experiments were analyzed by Western blotting with anti-hScrib (a), anti-Dlg1(b), anti-SNTB2 (c) and anti-TIP1 (d) antibodies. The upper panels show the bound proteins and the lower panels in each figure show the GST loading in Ponceau S staining. The bar graphs show the mean and standard deviation of the percentage of the total of protein bound, relative to the input, from at least three independent experiments. Student's t-test was calculated but no significance was observed.

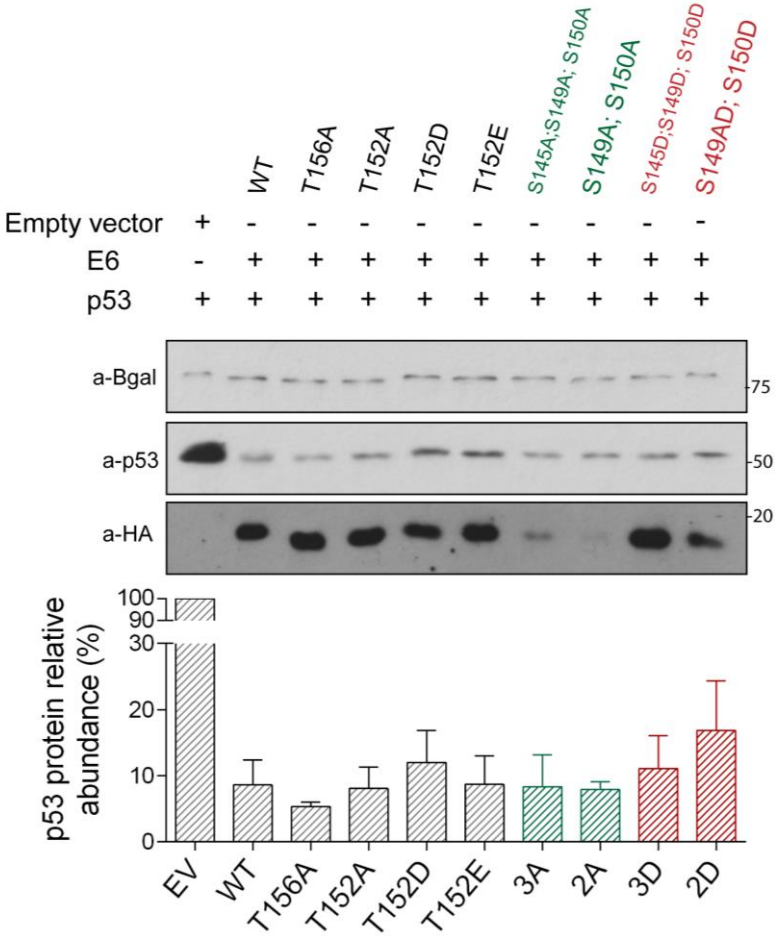


Figure 22.- HPV-16 E6 mutants upstream of the PBM do not affect degradation of p53. HPV-16 E6 HA fusion proteins were generated in pcDNA3.1 vector. p53 degradation assays with wild-type E6 and the corresponding mutants were performed in H1299 cells. H1299 cells were co-transfected with HA-16E6, wild-type p53 and β-galactosidase for 48 hours. Total cell extracts were collected and analyzed by western blot with DO-1, β-galactosidase, and anti-HA antibodies. The graph shows the percentage of the p53 protein relative abundance, relative to the p53 levels when it is co-transfected with the empty vector (First bar). The results shown correspond to the mean and the standard deviation from four independent experiments. Student's t-test analysis was performed but no significant difference was observed between the wild type and each mutant.

The S145, S149 and S150 serine residues affect HPV-16 E6 localisation.

Since phosphorylation by PKC has been previously shown to be involved in the nuclear-cytoplasmic trafficking of cellular proteins such as Dlg1 (O'Neill et al., 2011), the subcellular localization of the triple alanine/aspartate substitution mutants was examined by immunofluorescence microscopy of HEK293 cells transiently transfected with the pCDNA3.1-HA-16E6WT, pCDNA3.1-HA-16E63A and pCDNA3.1-HA-16E63D vectors. The quantification of the cytoplasmic/nuclear fluorescence intensity ratio was performed as described in Materials and Methods. The visual differences observed in Figure 23a show a tendency of the triple alanine substitution mutant to be more localized in the nucleus and the triple aspartate substitution mutant more localized in the cytoplasm. These results were confirmed by the quantification of the cytoplasmic/nuclear fluorescence intensity ratios (C:N) shown in Figure 23b. Note that the wild type have an average C:N ratio of 0.5 and the 3A mutant have statistically significant decreases in the C:N ratios compared to the wild-type, indicating a higher nuclear retention of this mutant. Conversely, the 3D mutant has an overall increase in the C:N ratios compared to the wild type, which indicates an increased cytoplasmatic retention.

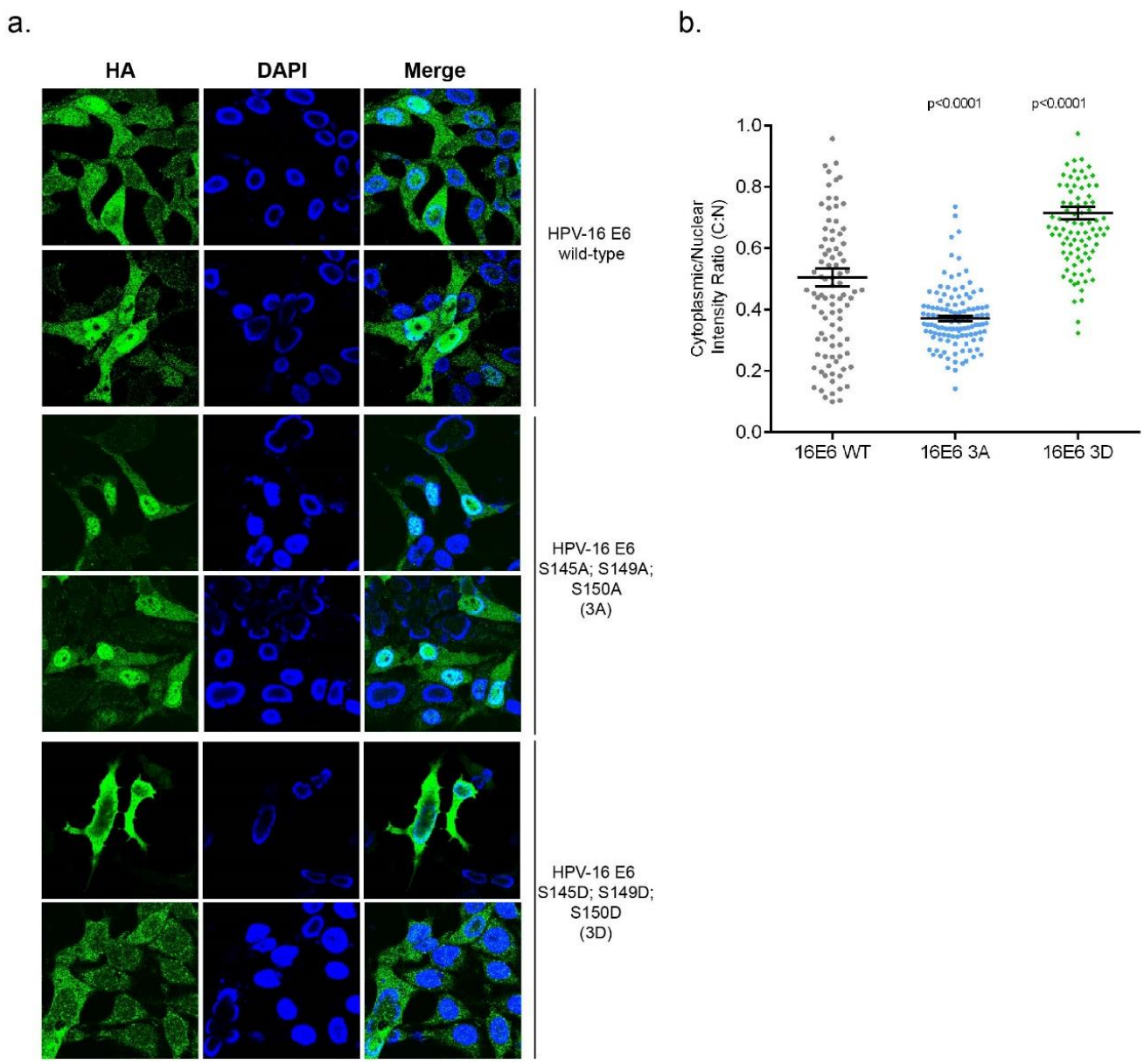


Figure 23.- The S145, S149 and S150 serine residues affect HPV-16 E6 localisation. HEK293 cells were transiently transfected with pCDNA3.1-HA-16E6 wild-type, pCDNA3.1-HA-16E6 (3A) and pCDNA3.1-HA-16E6 (3D) vectors, fixed after 48 hours and stained with an anti-HA (Green) antibody as well as DAPI (Blue). Representative fields are shown (a). (b) Quantification of the wild type 16E6 (grey), 16E6 3D (green) and 16E6 3A (blue) cytoplasmic/nuclear fluorescence intensity ratios (C:N). 50 individual cells were analyzed in three different experiments making a total of 150 cells. Unpaired t-test significance is shown ($p<0.0001$).

Preliminary results: The double and triple serine mutations upstream of the PBM differentially affect binding with additional E6 targets.

Since we have demonstrated that PKC phosphorylates E6 in multiple serine residues. We wanted to assess how the phospho-mimic and alanine substitution mutants of E6 could affect PKCα binding and one of its associated proteins, RACK1. Figure 24 shows that indeed, the phospho-mimic mutations in residues upstream of the PBM negatively regulate binding to PKC and significantly to RACK1. These two proteins have been previously reported to be involved in development of cancer. Indeed, it has been demonstrated that the PKCα-Dlg1 signaling pathway, positively affects migration and add to the pro-oncogenic role of Dlg1 (O'Neill et al., 2011)

Furthermore, interaction with PKCα, seems to be a possible prior event by which E6 gets phosphorylated by the same kinase. In fact, PKC phosphorylates the carboxy-terminal domain of lamin B2 protein, in a string of serine residues, which leads to almost complete inhibition of nuclear import (Hennekes et al., 1993). Furthermore, the NES1 of ZO-2 contains a serine 369 which gets phosphorylated by PKCε within the phosphorylation consensus site, mediating nuclear export of ZO-2 (Chamorro et al., 2009).

a.
a-RACK1
GST-E6-
GST-
Input 10%
GST
16E6 WT
16E6 dPBM
16E6 T156A
16E6 T152A
16E6 T152D
16E6 T152E
Input 10%
16E6 3A
16E6 2A
16E6 3D
16E6 2D
37
37
25
% of total
RACK1 bound
150
100
50
0
*
*
*
Input 10%
GST
WT
dPBM
T156A
T152A
T152D
T152E
Input 10%
3A
2A
3D
2D
b.
a-PKCa
GST-E6-
GST-
75
37
25
% of total
PKCa bound
200
150
100
50
0
c.
a-hScrib
GST-E6-
GST-
250
37
25
% of total
hScrib bound
300
200
100
0

Figure 24.- Phosphorylation of residues upstream foo HPV-16 E6 affect binding to PKC and RACK1. The HPV-16 GST E6 wildtype and mutant proteins were used in pull-down assays with the total cell extract from asynchronous HaCat cells. All pull-down experiments were analyzed by Western blotting with anti-RACK1 (a), anti-PKCα (b) and anti-hScrib (c) antibodies. The upper panels show the bound proteins and the lower panels in each figure show the GST loading visualized by Ponceau S staining. The bar graphs correspond to the mean and the standard deviation from three independent experiments. Student's t-test analysis (*$p<0.05$). Statistical significance was observed only in (a), the bars colored in red represent the mutants that are significantly different from the wild type.

Part 3: HPV-11 E6 affects the turnover of a phosphorylated active form of p53 in the presence of the DNA damaging agents.

The low-risk alpha HPV types have often been regarded as less potent versions of the high-risk HPV proteins, but with the ability to remain in the cell and be pathogenic, becoming one of the most widespread sexually transmitted diseases (Crow, 2012; Formana et al., 2012). Particularly, HPV-11 is the most common cause of the two most frequent benign tumours in the anogenital region and upper respiratory tract, but in some cases is detected in invasive cervical and anal cancer, but it is quite rare (Cornall et al., 2013; Guimerà et al., 2013; N. Li et al., 2011).

Although the low-risk E6 and E7 proteins do not drastically influence basal cell division as high-risk proteins, they can modulate the basal cell layer density at particular stages of the life cycle, such as during lesion formation. They can also mediate cell cycle reentry to restore competent environment for replication in the infected post-mitotic cells, but they do not drive cell division. Importantly, they also limit the differentiation of keratinocytes in the basal layer to retain a reservoir for infection and to drive differentiating cells into replication cells for genome amplification in the suprabasal layers (Doorbar et al., 2015; Gheit, 2019).

Furthermore, HPV-11 E6 associates with E6AP *in vivo* and degrades Bak through the low-risk HPV E6-E6AP complex (Thomas & Banks, 1999). The same complex is able to bind to the carboxy-terminal site of p53 but does not induce p53 degradation (Scheffner et al., 1993), mainly because p53 degradation depends on the E6 interaction with the core domain instead, as have been observed with high-risk types (Li & Coffino, 1996).

In homeostasis, the transcriptional activity of p53 is downregulated. By contrast, under stress, degradation and nuclear export of p53 are suppressed, and the nuclear import of p53 is enhanced, resulting in its nuclear accumulation (Gu & Zhu, 2012). Although many studies have focused on the roles of p53 under various stress stimuli and the different mechanisms used to achieve p53 induction (Meek, 2015); the basal non-induced levels of p53 have additional roles in regulating a range of essential genes in different cellular processes, one of them, particularly important for HPV life cycle, proliferation and differentiation of stem cells (Aloni-Grinstein et al., 2014; Meek, 2015; Solozobova, 2011).

Moreover, in normal homeostatic cells, p53 goes through post-translation modification cascades. But the effect of HPV in specific active forms of p53, has not been elucidated. Furthermore, the DNA damage response is the most studied mechanism which induces PTM of p53. From these events, the phosphorylation of p53 at Ser15, has been considered to be an initiating and nucleating event in p53 activation, that promotes the sequential modification of many subsequent

residues (Saito et al., 2003; Saito et al., 2002; Sakaguchi et al., 1998, 2000). Most of these modifications have the same outcome, dissociation of Mdm2 which results in p53 stabilization (Shieh et al., 1997).

Accordingly with a recent report showing that HPV-11 E6 degrades p53 under high-confluency conditions (Murakami et al., 2019). We aimed to assess the effect of HPV-11 E6 in the turn-over of wild type p53 in the presence of DNA damaging agents. We show that wild type p53 is degraded by 11E6 in the presence of Etoposide. Correspondingly, the p53 S15D mutant, which mimics the phosphorylated state of p53 in the presence of Etoposide, is also degraded by 11E6. Furthermore, 11E6 co-localizes with p53 in the presence of DNA damaging agents and with the S15D mutant.

The low-risk type HPV-11 E6 protein degrades the wild-type p53 protein in the presence of DNA damaging agents.

In order to assess if HPV-11 E6 could have carcinogenic properties in specific stress conditions, we evaluated if 11E6 could degrade p53 in H1299 cells, which are a human non-small cell lung carcinoma immortalized cell line derived from the lymph node (Giaccone et al., 1992). These cells have a homozygous partial deletion of the TP53 gene and do not express the tumour suppressor p53 protein. After optimizing the transfection of p53 and 11E6, we treated the transfected cells with Etoposide, Teniposide, H_2O_2 and Nocodazole. The results in Figure 25 show that p53 levels were reduced by 11E6 in the presence of Etoposide, Teniposide and H_2O_2, although Etoposide was the most effective.

Etoposide is a semisynthetic derivative of podophyllotoxin, which is a substance extracted from the mandrake root *Podophyllum peltatum* (Montecucco et al., 2015). It is cell cycle dependent and phase specific, acting primarily in the G2 and S phases of the cell cycle. It binds to and inhibits mainly the topoisomerase II alpha isoform, disrupting its binding to cleaved DNA molecules, preventing rejoining of single and double strand DNA breaks (Pommier et al., 2010). This causes critical errors in DNA synthesis at the premitotic stage of cell division, leading to apoptosis of the cancer cell. Likewise, Teniposide is a semisynthetic derivative of podophyllotoxin with antineoplastic activity (Clark & Slevin, 1987). It acts in the late S or early G phase of the cell cycle and forms a ternary complex with the enzyme topoisomerase II and the DNA, resulting in dose-dependent single- and double-stranded breaks in the DNA. Etoposide and Teniposide are used

in the therapy of several forms of solid tumors, leukemia and lymphoma, usually in combination with other agents (Pommier et al., 2010).

Hydrogen peroxide (H_2O_2) is a weak acid with strong oxidizing properties and is a well-documented component of living cells. It plays major roles in host defense and oxidative biosynthetic reactions and, at low levels, it can also function as a signaling agent (Halliwell et al., 2000). H_2O_2 has been involved in modulating both contractile and growth-promoting pathways. Furthermore, hydrogen peroxide can have a role in cancer development. Indeed, cancer cells produce high amounts of H_2O_2, and this increase has been linked to many trademarks signs of cancer, including DNA damage induction, cell proliferation, apoptosis resistance, metastasis, angiogenesis and hypoxia-inducible factor 1 activation (Szatrowski & Nathan, 1991).

Nocodazole is a synthetic tubulin-binding agent with antineoplastic activity. It binds to beta-tubulin and disrupts microtubule assembly and disassembly dynamics, preventing mitosis and inducing apoptosis in tumor cells (Vasquez et al., 1997).

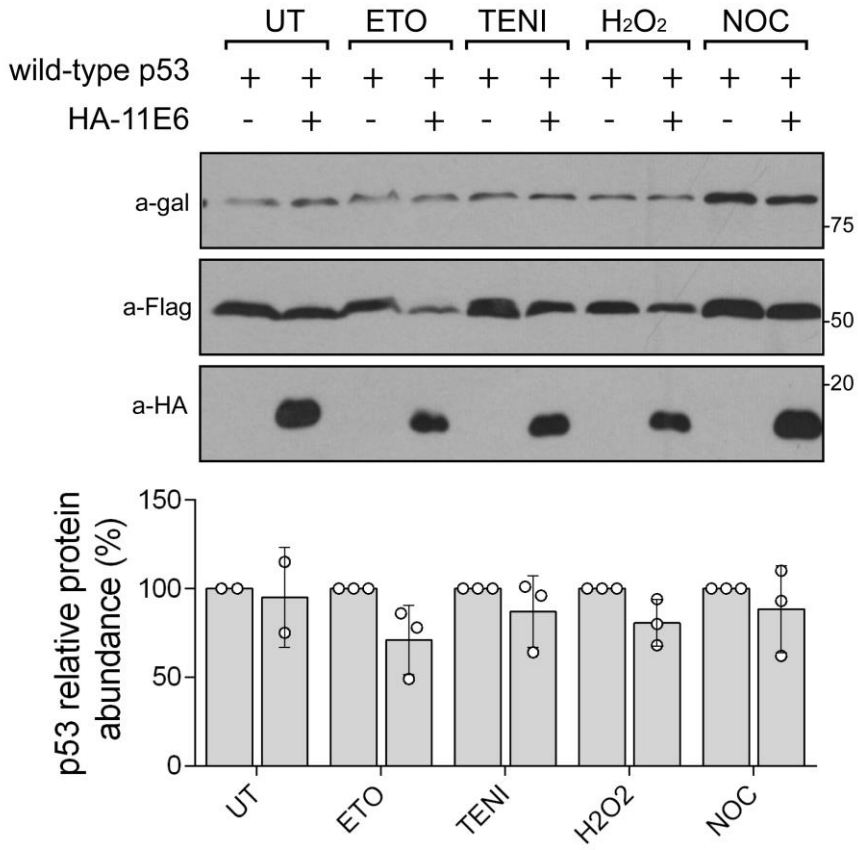


Figure 25. – HPV-11 E6 can degrade p53 in the presence of DNA damaging agents. The p53 degradation assay by 11E6 protein was done by co-transfecting Flag-p53 and HA-E611 in H1299 cells. β-galactosidase was transfected as a transfection efficiency control. The cells were transfected for 48 hours and treated with each DNA damage agent 24 hours post transfection and for 16 hours. Concentrations are given in the Material and Methods. Whole lysates were taken after 48 hours and probe with anti-Flag, anti-HA, and β-galactosidase antibodies. The bar graph represents the percentage of the relative abundance of p53. The bars represent the mean and the error bars the standard deviation from three independent experiments for each treatment. The dots represent the results of each experiment. Student's t-test significance was calculated, but no significance was obtained.

The turnover of wild-type p53 is affected in the presence of HPV-11 E6, in H1299 cells treated and not treated with Etoposide.

Etoposide induces phosphorylation of p53 at serine 15, through the ATM and ATR kinases (Appella & Anderson, 2000; Saito et al., 2002). Indeed, it has been described that the activation of p53 occurs within 30 minutes of the DNA damage stimulus, and although the ATM activation can be transient, the subsequent slower activation of ATR might occur, providing continued Ser15 phosphorylation that can endure for several hours after the initial stimulus. Considering this, we performed half-life experiments with cycloheximide for a period of 2 hours after treatment with etoposide. The whole cell lysates were collected every 30 minutes after cycloheximide induction, and p53 levels were observed in the presence of 11E6 or the empty vector control. We observed that ectopic expression of wild type p53, co-transfected with the empty vector, maintains steady state levels, which correlates with the transient transfection experiments. Furthermore, when wild type p53 transfected cells are treated with etoposide, we observed stabilization of p53, which would be expected following induction of DNA damage. When 11E6 was co-transfected with p53, we did not see any difference in the untreated cells, whereas in the presence of etoposide, the p53 half-life was significantly reduced (Figures 26 and 28a).

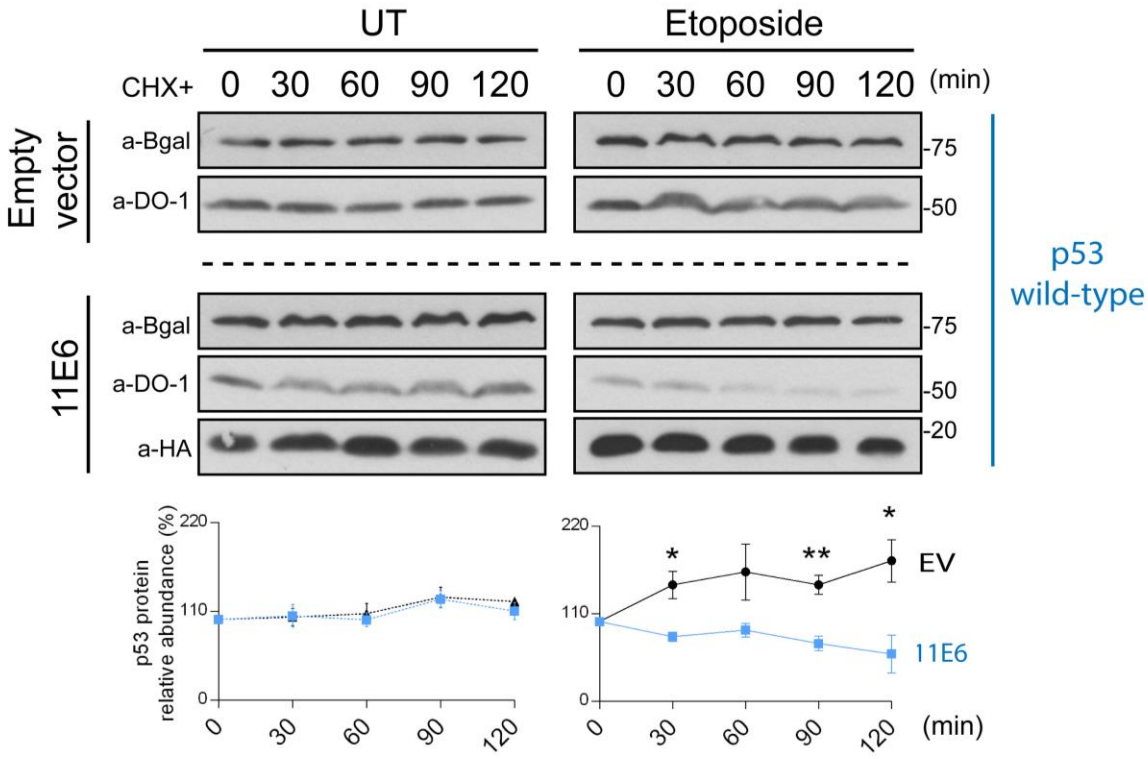


Figure 26.- Evaluation of the half-life of exogenously expressed wild type p53 in H1299 cells, treated and non-treated with Etoposide, in the presence and absence of exogenously expressed 11E6. The time course of cycloheximide treatment after etoposide treatment is shown. Whole lysates were taken every 30 minutes for 2 hours and probe with anti-p53 (DO-1) antibody, anti-HA, and β-galactosidase antibodies. β-galactosidase was used a transfection efficiency control. The lower dot graphs represent the percentages of the relative abundance of p53, obtained based on the p53:βgal ratio. The line represents the tendency throughout the half-life. The dots symbolize the mean and the error bars the standard deviation, from three independent experiments. Student's t-test significance was calculated for each time-point (*p<0.05, **p<0.01).

HPV-11 E6 induces degradation of the active phospho-mimic form of p53, S15D independently of DNA damage.

Since serine 15 of p53 is phosphorylated in response to Etoposide-induced DNA damage, we obtained the phospho-mimic mutant and the alanine substitution mutant of p53 unable to be phosphorylated, S15D and S15A, respectively. (Loughery et al., 2014). The results in figure 27a show the half-life of S15D, the phospho-mimic mutant of p53, with and without Etoposide treatment, in the presence and absence of 11E6. In the absence of 11E6 and after Etoposide treatment there is an increase of S15D p53 (Figure 28b), probably as a result of its inherent inability to not be negatively regulated by Mdm2. Furthermore, other residues, independent of serine 15's nucleating effect, may be phosphorylated and positively regulate p53 upon DNA damage. It is possible to assume that the S15D mutant might be more stable than the wild type p53 in the half-life experiments, although we have not performed the experiment. By contrast, when S15D is co-transfected with 11E6, its relative abundance is significantly down in cells collected after 30, 60, 90 and 120 minutes post-cycloheximide treatment, both in untreated cells and in cells treated with Etoposide, where the effect is even more evident (Figure 27a and 28b). This confirms that 11E6 steadily degrades the stabilized form of p53, dependent on phosphorylation of serine 15 after etoposide induction. Whether other residues are being multiply phosphorylated by etoposide and also contributing to 11E6-induced degradation, needs to be further clarify.

Additionally, we repeated the half-life experiments with the alanine substitution mutant S15A (Figures 27b and 28c). The relative abundance percentage of S15A does not change in untreated or etoposide-treated cells, neither in the presence or absence of 11E6. Importantly, the fact that we do not see degradation after etoposide treatment and 11E6 co-transfection, confirms that p53 phosphorylated on serine 15 is a target of 11E6 for turnover control.

All statistical results are shown in figure 28, where empty vector and 11E6 curves are superimposed.

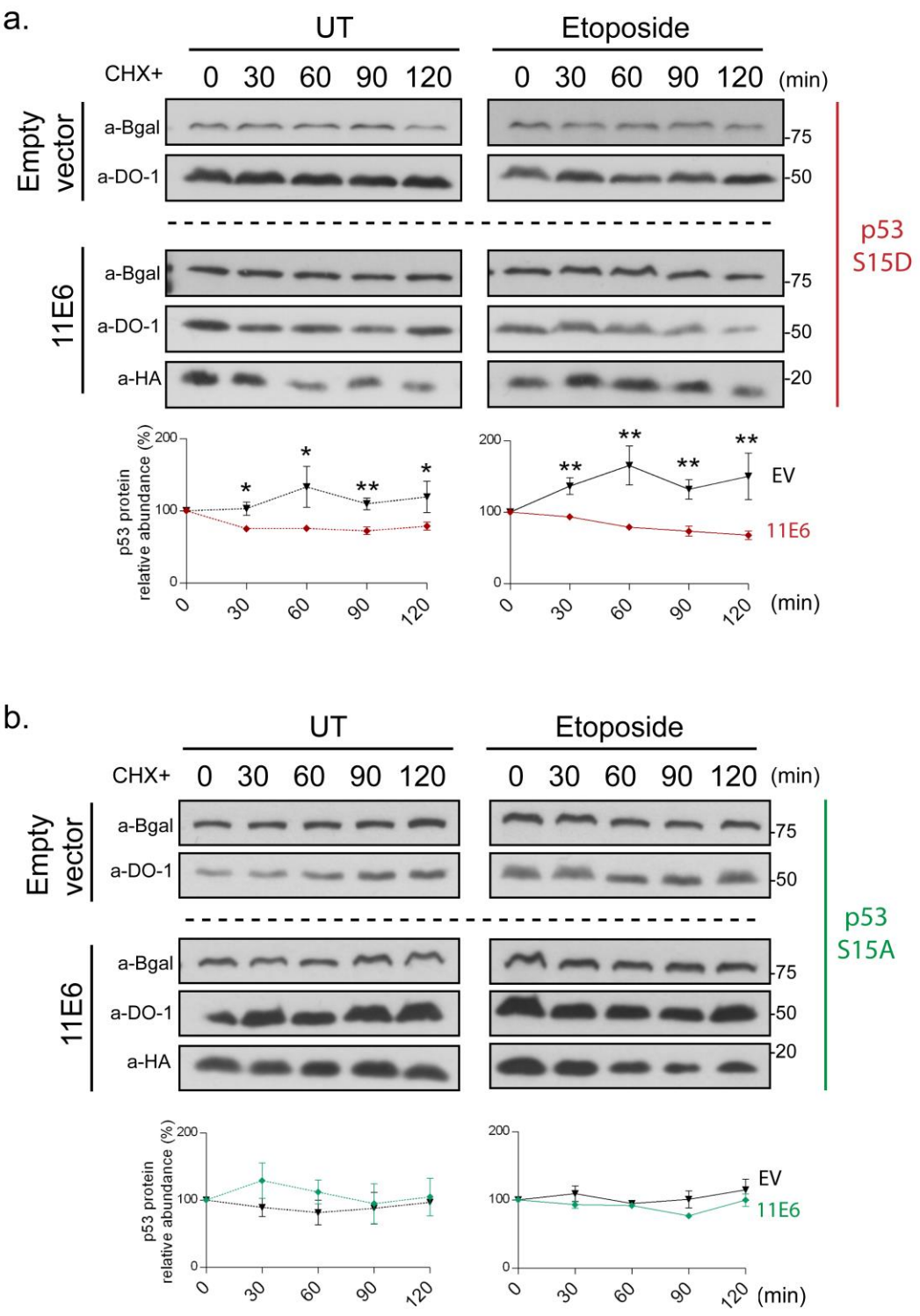
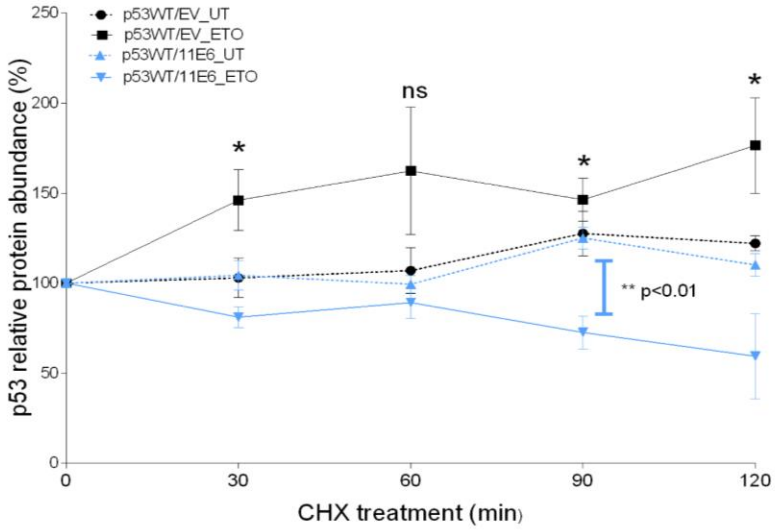
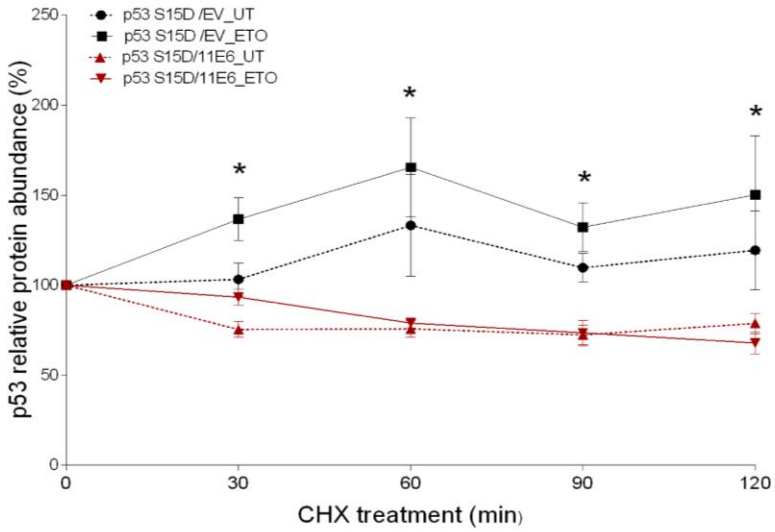

a.
UT
Etoposide
CHX+
0 30 60 90 120
0 30 60 90 120 (min)
Empty vector
a-Bgal
a-DO-1
75
50
11E6
a-Bgal
a-DO-1
a-HA
75
50
20
p53 S15D
p53 protein relative abundance (%)
200
100
0
*
**
EV
11E6
0 30 60 90 120 (min)
b.
UT
Etoposide
CHX+
0 30 60 90 120
0 30 60 90 120 (min)
Empty vector
a-Bgal
a-DO-1
75
50
11E6
a-Bgal
a-DO-1
a-HA
75
50
20
p53 S15A
p53 protein relative abundance (%)
200
100
0
EV
11E6
0 30 60 90 120 (min)

Figure 27.- Evaluation of the half-life of exogenously expressed S15D and S15A p53 mutants in H1299 cells, treated and non-treated with Etoposide, in the presence and absence of exogenously expressed 11E6. (a and b) The time course of cycloheximide treatment after etoposide treatment is shown. Whole lysates were taken every 30 minutes for 2 hours and probe with anti-p53 (DO-1) antibody, anti-HA, and β-galactosidase antibodies. β-galactosidase was used a transfection efficiency control. The lower dot graphs represent the percentages of the relative abundance of p53, obtained based on the p53:βgal ratio. The line represents the tendency throughout the half-life. The dots symbolize the mean and the error bars the standard deviation, from three independent experiments. Student's t-test significance was calculated for each time-point (*$p<0.05$, **$p<0.01$).

a.


p53WT/EV_UT
p53WT/EV_ETO
p53WT/11E6_UT
p53WT/11E6_ETO
p53 relative protein abundance (%)
CHX treatment (min)
ns
*
*
*
** p<0.01
0
30
60
90
120
0
50
100
150
200
250

b.


p53 S15D /EV_UT
p53 S15D /EV_ETO
p53 S15D/11E6_UT
p53 S15D/11E6_ETO
p53 relative protein abundance (%)
CHX treatment (min)
*
*
*
0
30
60
90
120
0
50
100
150
200
250

c.

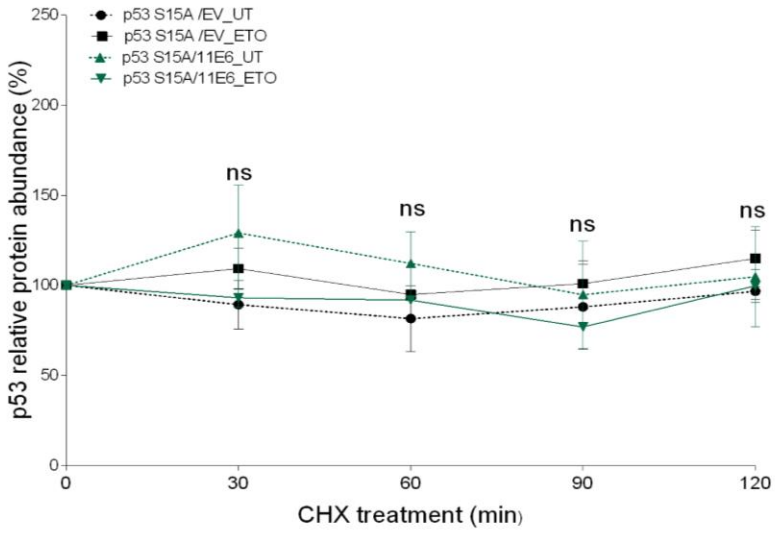

p53 S15A /EV_UT
p53 S15A /EV_ETO
p53 S15A/11E6_UT
p53 S15A/11E6_ETO
p53 relative protein abundance (%)
CHX treatment (min)
ns
ns
ns
ns
0
30
60
90
120
0
50
100
150
200
250

Figure 28.- Super imposition of the p53 relative abundance percentage curves based in the half-life of p53 wild-type and mutants, with and without etoposide treatment, in the presence of exogenously expressed 11E6 in H1299 cells. (a, b and c) The dots and lines graphs represent the percentages of the relative abundance of p53, obtained based on the p53:βgal ratio. The line represents the tendency throughout the half-life. The dots symbolize the mean and the error bars the standard deviation, from three independent experiments. Student's t-test significance was calculated for each time-point (*p<0.05, **p<0.01).

HPV-11 E6, wild type and mutants p53 cellular localization in untreated H1299 cells.

Having found that HPV-11 E6 can specifically target active forms of p53, we proceeded to investigate this further by immunofluorescence, and whether this was reflected in the level of co-localisation.

We initially assessed the localization of exogenously expressed 11E6 and p53 in H1299 cells separately (Figure 29a). In contrast to what is observed with high-risk E6 proteins, 11E6 is mostly localized in the cytoplasm of the cell (Figure 31). In the case of wild type p53, it has a nuclear localization as expected (Figure 29b and 31). The p53 phospho-mimic mutant has a higher cytoplasmic localization than wild type p53, whereas the alanine substitution mutants mostly maintain its nuclear localization (Figure 30a and 31). Furthermore, when 11E6 is co-transfected, we observed wild type p53 redistribution to the cytoplasm (Figure 29c and d). Subsequently, we evaluated the effect on both p53 mutants, S15D and S15A, co-transfected with 11E6. As observed in Figure 30a and 31, the S15D shows a decrease in cytoplasmic localisation, possibly due to 11E6 degradation occurring in the cytoplasm, while the S15A behaves similar to the wild type and no significant effect in localisation was observed in the presence of 11E6.

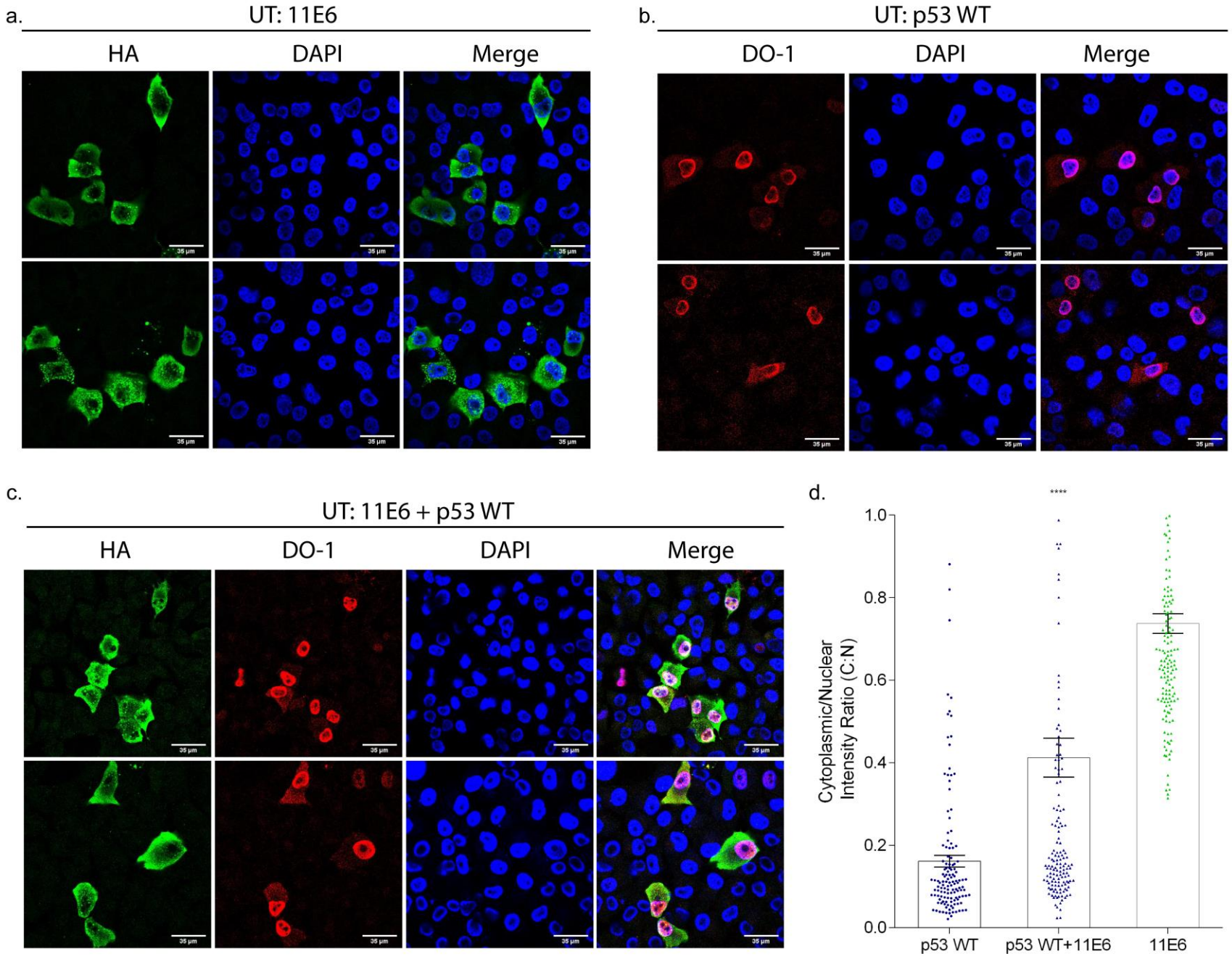

a.
UT: 11E6
HA
DAPI
Merge
b.
UT: p53 WT
DO-1
DAPI
Merge
c.
UT: 11E6 + p53 WT
HA
DO-1
DAPI
Merge
d.
****
Cytoplasmic/Nuclear Intensity Ratio (C:N)
1.0
0.8
0.6
0.4
0.2
0.0
p53 WT
p53 WT+11E6
11E6

Figure 29.- HPV-11 E6 affects the localisation of wild type p53. H1299 cells were transiently transfected with 11E6 and wild type p53, fixed after 48 hours and stained with an anti-HA (Green) antibody, anti-p53/DO-1 (red) as well as DAPI (Blue). Representative fields of single transfection experiments of 11E6 and wild type p53 are shown in (a) and (b), respectively. (c) Representative fields of the co-transfection of 11E6 and wild type p53 is shown. (d) The cytoplasmic/Nuclear Intensity Ratio (C:N) of p53 in the presence of 11E6 was calculated based on the intensity of each cellular compartment. The wild type p53 has an average C:N ratio of 0.2 indicating nuclear localization. Ratios above 0.2 show a tendency for cytoplasmic localisation. The dots represent the C:N ratio for each cell. The bars represent the mean and the error bars the standard error of the mean. Student's t-test significance was calculated for each time-point (****$p<0.0001$). Significant differences were observed between the wild type p53 ratios in the presence and absence of 11E6, suggesting higher cytoplasmic localisation in the presence of 11E6.

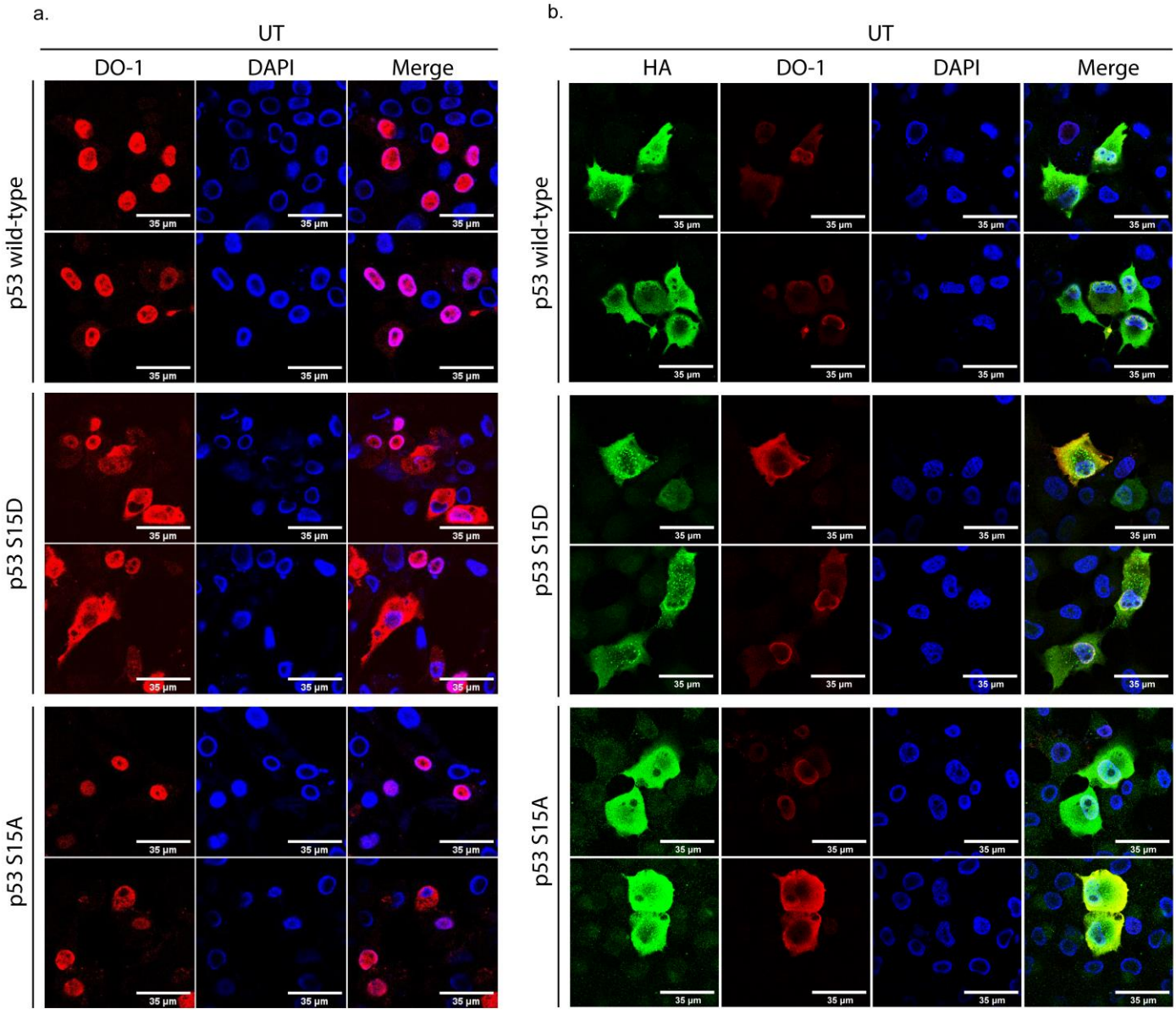


Figure 30.- HPV-11 E6 co-localizes with S15D phospho-mimic mutant of p53. H1299 cells were transiently transfected with wild type p53, S15D and S15A p53 mutants, fixed after 48 hours and stained with anti-p53/DO-1 (red). (a) Single transfection of wild type p53, S15D and S15A mutants. (b) Co-transfection of 11E6 and wild type p53, S15D and S15A mutants. Representative fields are shown.

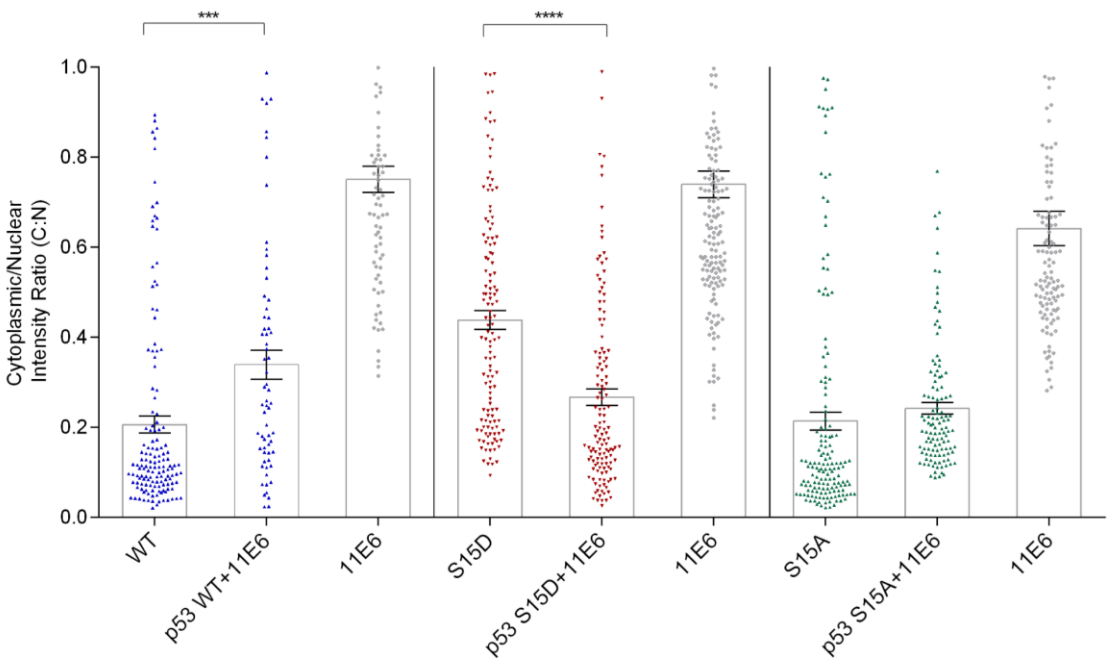


Figure 31.- Effect of HPV-11 E6 in the localisation of wild-type p53 and its corresponding S15 mutants. Statistical analysis of wild type, S15D and S15A mutant p53 forms in the absence and presence of 11E6. The cytoplasmic/Nuclear Intensity Ratio (C:N) was calculated based on the intensity of each cellular compartment. The figure analysis was performed with FiJi. The wild type p53 has an average C:N ratio of 0.2 indicating nuclear localization. Ratios above 0.2 show a tendency for cytoplasmic localisation. The dots represent the C:N ratio for each cell. The bars represent the mean and the error bars the standard error of the mean. Student's t-test significance was calculated for each time-point (****p<0.0001; ***p<0.001).

HPV-11 E6 specifically colocalizes with wild-type p53 and the active form of p53 upon DNA damage stimulus with Etoposide treatment.

Since 11E6 induces the re-distribution of p53 to the cytoplasm, but probably degrades mostly the cytoplasmic S15D form, we aimed to assess the levels of colocalization of 11E6 with wild type and the mutant form of p53 in the absence and presence of Etoposide. We firstly evaluated wild type, S15D and S15A p53 forms in the context of single transfection, upon treatment with

Etoposide (Figure 32a). We observed mostly nuclear localisation of wild type p53 and S15A, but higher cytoplasmic localisation of S15D as previously shown.

When 11E6 was co-transfected, we mostly observed co-localization of 11E6 with wild type p53 and S15D upon etoposide treatment (Figure 32b). Furthermore, co-localization is clearly observed in the cytoplasm (etoposide merge panel). Therefore, 11E6 co-localizes with the p53 S15D mutant both in the untreated and in the etoposide treated H1299 cells. In contrast, the S15A mutant resembles the wild type phenotype in the untreated conditions but following etoposide treatment there is still no co-localisation with 11E6. These results indicate that phosphorylation of p53 at S15 induces a change in p53 which allows 11E6 to interact and target it for degradation in the cytoplasm.

We further calculated the Pearson's colocalization coefficient of 11E6 and S15D p53 mutant (Figure 33 a and b). Indeed, we observed that 11E6 colocalizes significantly with S15D mutant in untreated conditions in the cytoplasm. This is in contrast with previous results that indicate that phosphorylation at serine 15 masks a nuclear export signal and contributes to the retention of p53 within the nucleus . We have observed in all experiments that colocalization mostly happens in the cytoplasm, where the S15D mutant is highly distributed and where it colocalizes with 11E6, which subsequently degrades it. Whether 11E6 firstly induces wild type p53 cytoplasmic localisation, which induces p53 phosphorylation and further 11E6-induced degradation needs to be further investigated with endogenous cellular models.

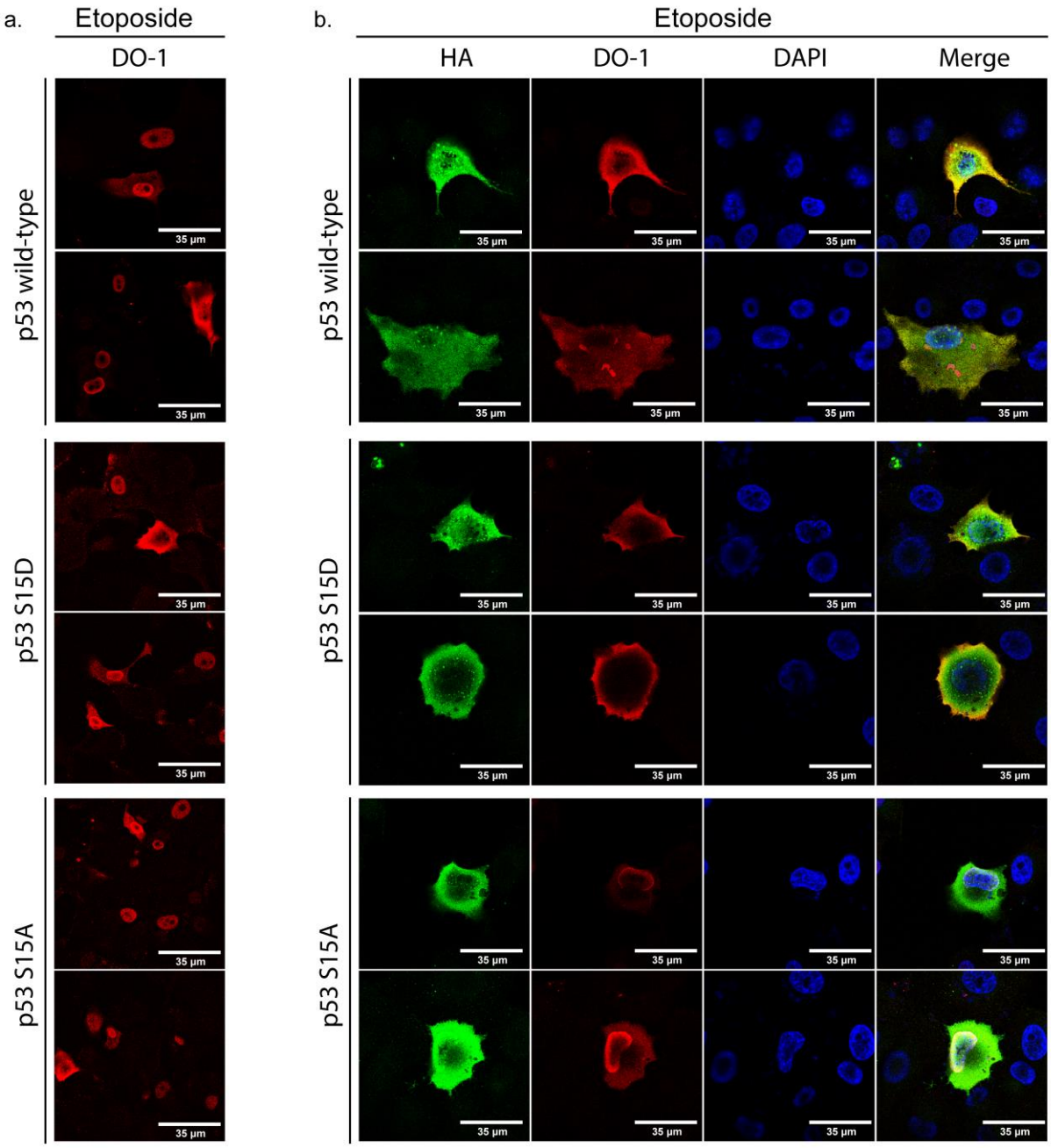


Figure 32.- HPV-11 E6 colocalizes with wild type p53 under stress conditions. H1299 cells were transiently co-transfected with wild type p53. S15D and S15A p53 and 11E6, fixed after 48 hours and stained with anti-HA (green), anti-p53/DO-1 (red) and DAPI. Representative fields are shown. (a) Single transfection of wild type p53, S15D and S15A mutants. (b) Co-transfection of 11E6 and wild type p53, S15D and S15A mutants. Representative fields are shown.

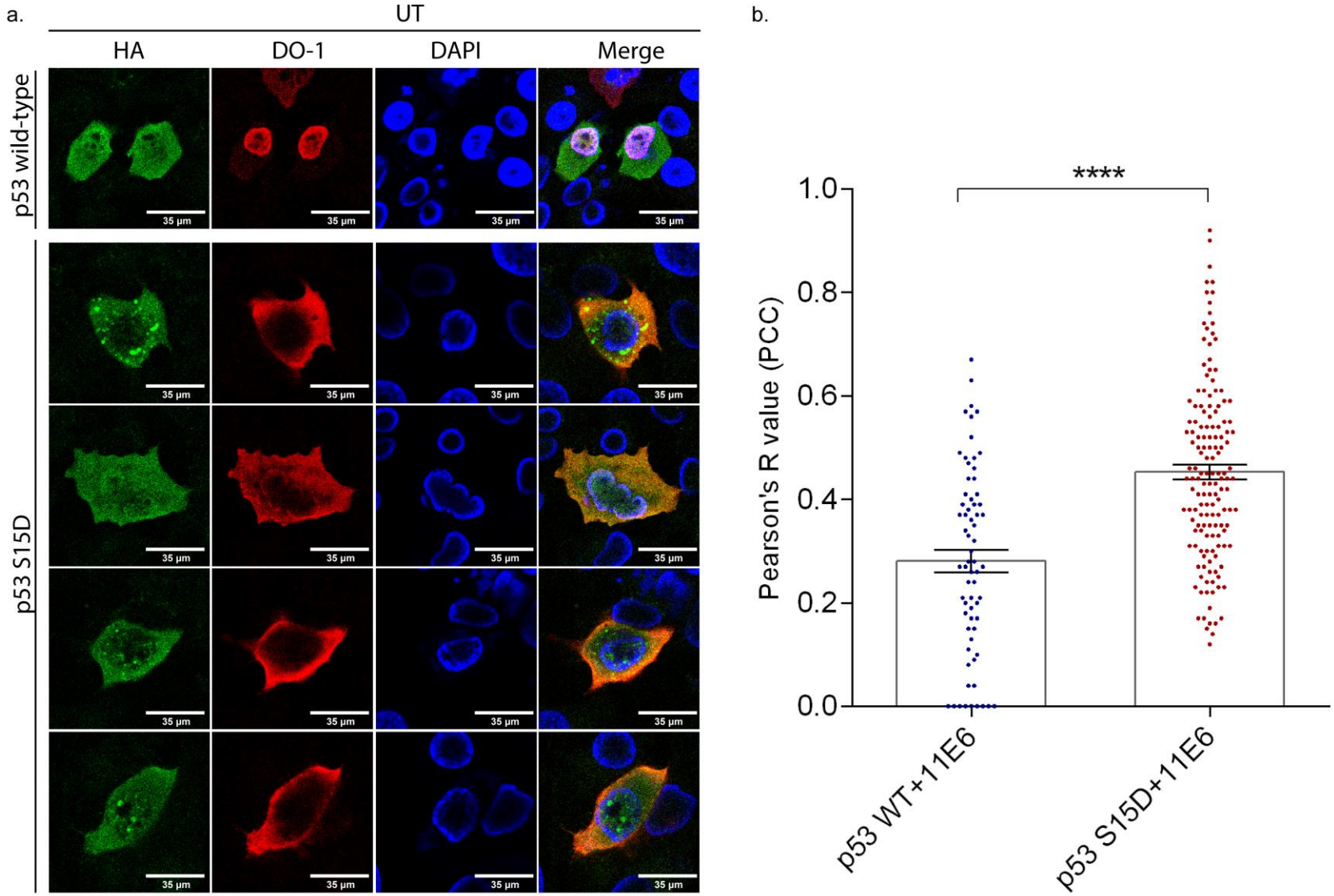

a.
UT
HA
DO-1
DAPI
Merge
p53 wild-type
p53 S15D
35 µm
b.
****
Pearson's R value (PCC)
1.0
0.8
0.6
0.4
0.2
0.0
p53 WT+11E6
p53 S15D+11E6

Figure 33.- HPV-11 E6 colocalizes with the activated form of p53, S15D. (a) H1299 cells were transiently co-transfected with wild type p53. S15D and S15A p53 and 11E6, fixed after 48 hours and stained with anti-HA (green), anti-p53/DO-1 (red) and DAPI. Representative fields are shown. Left panel: Untreated. Right panel: Etoposide treatment. (b)The person's colocalization coefficient was calculated using the FiJi programme (Rueden et al., 2017). The statistical analysis was based on 100 cells in three separate experiments. Unpaired t test significance ($p<0.0001$).

HPV-11 E6 colocalizes with wild type p53 in the presence of other types of DNA damaging agents.

In order to corroborate if indeed that general DNA damage response stimulates the co-localization of 11E6 with p53 (Figure 34), we treated H1299 cells single-transfected with wild type p53 and the corresponding mutants with Teniposide and Thymidine, which are known DNA damaging agents and other genotoxic stress-inducing agents: H_2O_2 and Nocodazole. As with Etoposide, we observed that treatment with H_2O_2 and Thymidine does not induce re distribution of p53 to the cytoplasm (Figure 34). Furthermore, when 11E6 was co-transfected with p53, we observed more cytoplasmic distribution and also co-localisation with 11E6 in the case of H_2O_2 and Teniposide (Figure 35). Indeed, H_2O_2 and Teniposide generate activated forms of p53 by phosphorylation, although serine 15 may not be the main and only targeted residue. Furthermore, it is not possible to consider the mono-phosphorylated form of p53 as an activated form, since p53 is a phosphoprotein with multiple phosphorylation events happening in different cascades, which can initiate with a determined kinase, depending on the homeostatic process, the cell cycle or stress-induced mechanisms.

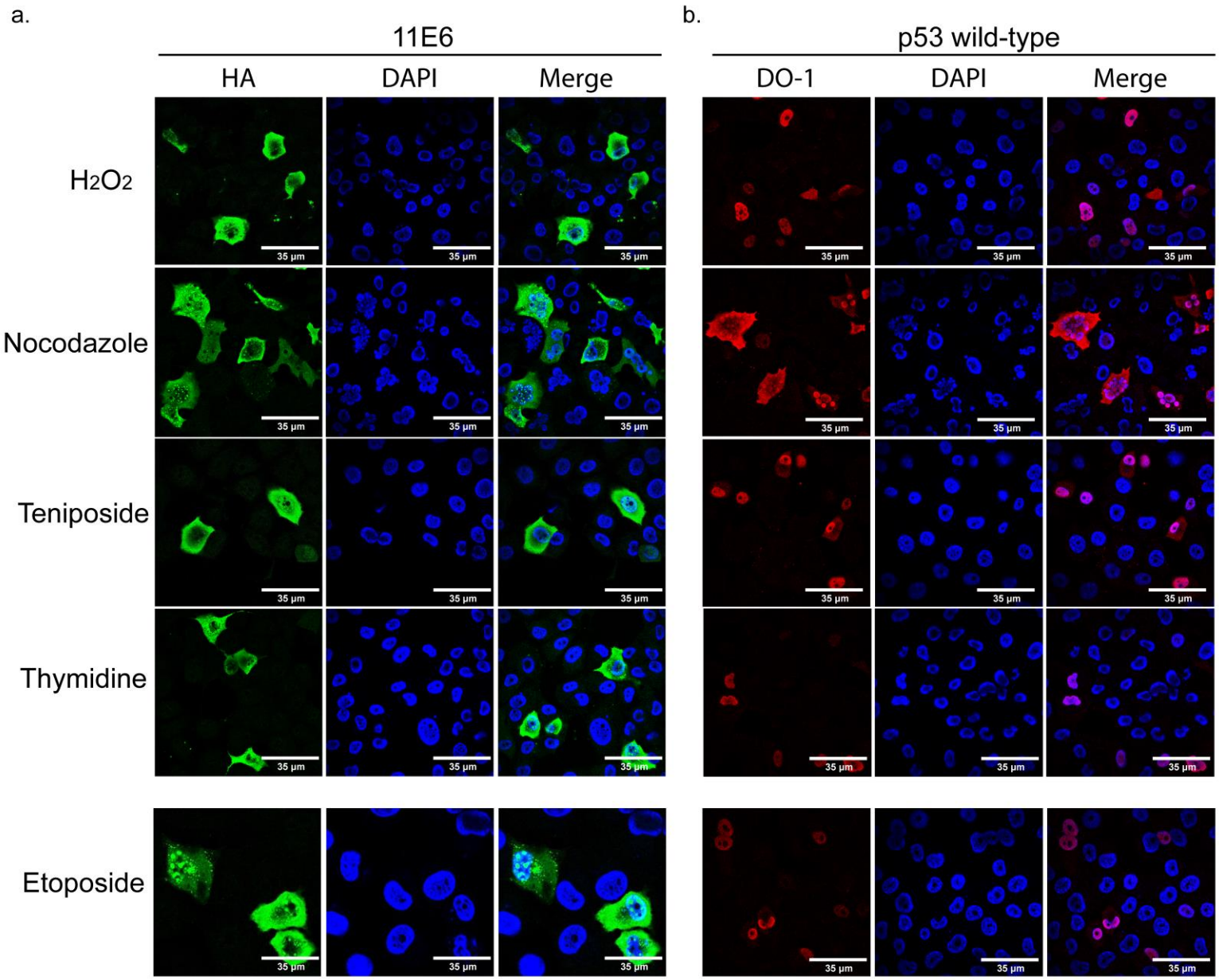

a.
11E6
HA
DAPI
Merge
b.
p53 wild-type
DO-1
DAPI
Merge
H2O2
Nocodazole
Teniposide
Thymidine
Etoposide
35 µm

Figure 34.- Effect of DNA damaging agents on HPV-11 E6 and p53. H1299 cells were transiently transfected with wild type p53 and 11E6, separately, and treated after 24 hours with Etoposide, H_2O_2, Nocodazole, Teniposide and Thymidine. After48 hours post-transfection, the cells were stained with anti-HA (green), anti-p53/DO-1 (red) and DAPI. Representative fields are shown.

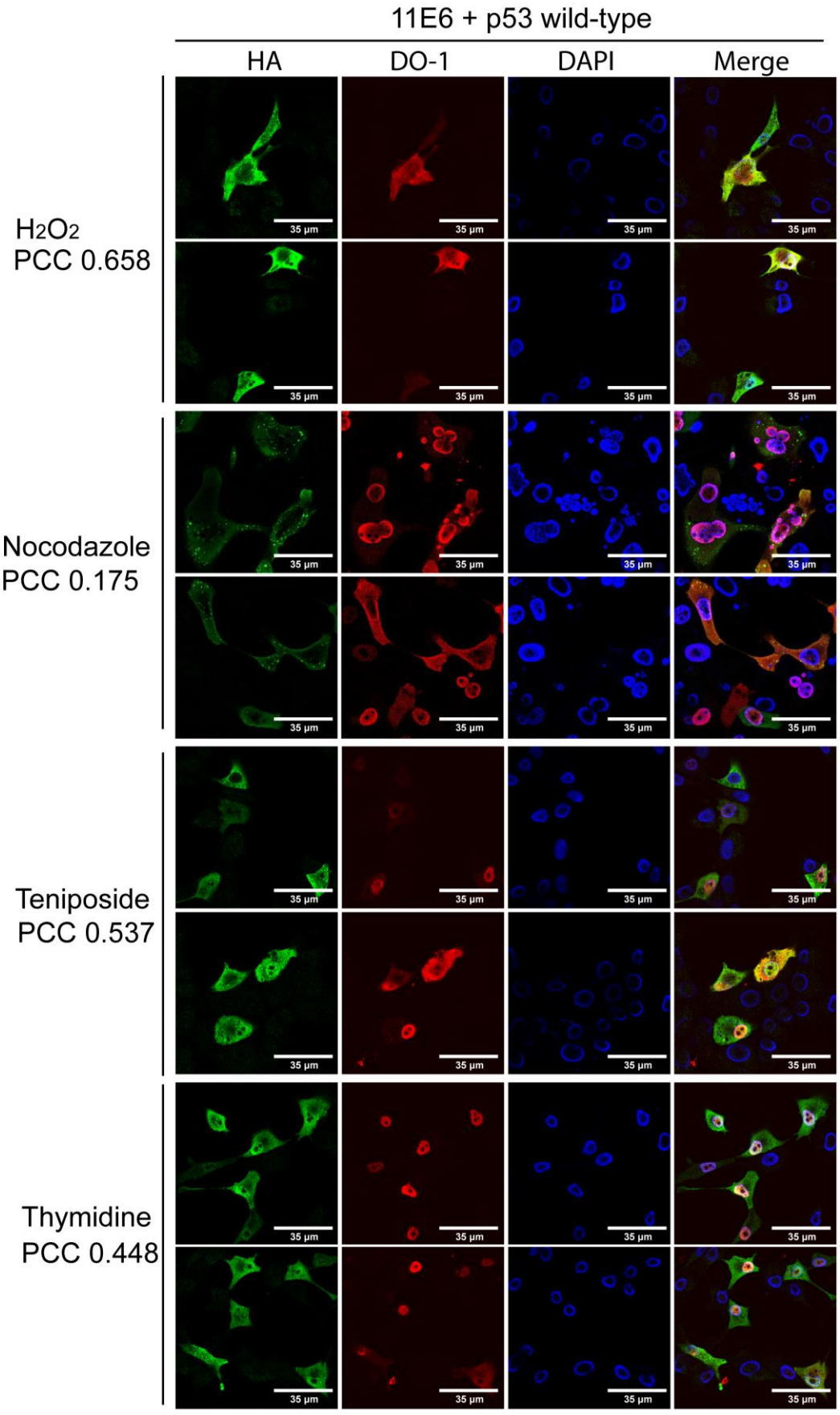

11E6 + p53 wild-type
HA
DO-1
DAPI
Merge
H2O2
PCC 0.658
Nocodazole
PCC 0.175
Teniposide
PCC 0.537
Thymidine
PCC 0.448
35 µm

Figure 35.- Effect of DNA damaging agents on cells co-transfected with in HPV-11 E6 and p53. H1299 cells were transiently co-transfected with wild type p53. and 11E6, and treated after 24 hours with H_2O_2, Nocodazole, Teniposide and Thymidine. After48 hours post-transfection, the cells were stained with anti-HA for 11E6 (green), anti-p53/DO-1 (red) and DAPI. Representative fields are shown, with their respective Pearson's colocalization coefficients.

CHAPTER 4: DISCUSSION

PART 1

It has been previously reported that the number of PDZ targets bound and the promiscuity of the E6 PBM is an oncogenic trait (James & Roberts, 2016b; Thomas et al., 2016). Based on this, we show that there is a correlation between the susceptibility of the E6 protein to be phosphorylated by PKA, and the functional diversity and flexibility of E6 in binding to PDZ targets. It is well known that the alpha HPV types, including the high-risk and low-risk types differ, from each other by more than 10% in the sequence of the conserved L1 gene, and that expansion of the population, geographical dispersion and time have driven evolution through single-nucleotide variation into variants, sub-variants and isolates that differ in their risk of persistence and carcinogenic potential (Chen et al., 2005; Hildesheim et al., 2001; Schiffman et al., 2009). Knowing this, it is possible to say that these single nucleotide variations among high-risk species groups have accumulated and fixed in the carboxyl-terminus of the E6 protein, resulting in the phylogenetic distribution and risk classification that is observed now; and that these sequence changes might have been driven by the interactions with PDZ proteins.

This assumption strongly correlates with the evolving mechanism of the PDZ-PBM interactions in biological systems, via 'rewiring of interactions'. The formation of linear motifs allowed PDZ domain-mediated interactions to form multi-structural protein conformations, thus contributing to increase the chance of acquiring sequence mutations on the carboxyl-terminus sequence of the PDZ ligands during evolution (Kim et al., 2012). Considering this, it is possible to say that HPV PBMs have evolved residues important for binding several PDZ proteins that eventually have conferred a higher binding promiscuity to high-risk types. Moreover, cellular PBM proteins show tissue-specific expression patterns that allow the formation of tissue-specific cell signaling complexes. Therefore, like the one-third of all human PDZ ligands that obtained their PBMs via carboxyl-terminus sequence mutations (Hung & Sheng, 2002; Kim et al., 2012), the E6 proteins might have evolved a PBM in order to acquire a competitive advantage in a specific cellular environment; using PDZ domain-mediated interactions to help them maintain their genomes in the cell, while coincidentally giving them the ability to transform cells (Nguyen et al., 2003). Additionally, the acquired mutations might have modified not only the core PBM, but also upstream residues that could, potentially, have conferred a consensus phosphorylation motif on the protein; generating the capability for new interactions and for forming new complexes, as has been observed with the phosphorylated E6 proteins (Boon et al., 2015; Boon & Banks, 2013; Lee & Zheng, 2010a; Nourry et al., 2003; Pim et al., 2012). The results show that the residues immediately upstream of the HPV-18 E6 PBM that confer phosphorylation are also important for

binding and recognition of multiple PDZ proteins, indicating that the capacity of high-risk E6 proteins to be phosphorylated is also linked to its functional flexibility.

The phosphorylation consensus sequence embedded within the PBM of high-risk E6 proteins, represents an oncogenic trait

As has been shown before, the cancer-associated genotypes from high-risk group 1 interact with more PDZ proteins than the possibly cancer-associated ones in Groups 2A and 2B, independent of the presence of an intact core PBM, as is the case of HPV-66 and HPV-40 E6 (Thomas et al., 2016). We demonstrate that there is a correlation between the promiscuity and functional flexibility of the carboxyl-terminus of E6 proteins, and their ability to be phosphorylated. The low-risk type HPV-40, which is not phosphorylated by PKA, AKT or CHK1, only bound hDlg1, whilst HPV-66, which is also very rarely associated with cancer, also had a very restricted pattern of PDZ recognition and was also not phosphorylated by any of these kinases. In contrast, the high-risk carcinogenic HPV-18 can bind multiple PDZ targets and is strongly phosphorylated by all three kinases (Boon & Banks, 2013; Kühne et al., 2000; Thatte et al., 2018; Thomas et al., 2016). Therefore, this suggests that having a kinase recognition motif within the PBM might represent an additional oncogenic trait, which directly affects both the identity of the kinases that phosphorylate E6, the efficiency of that phosphorylation, and at the same time directly contributes towards PDZ recognition in the absence of phosphorylation.

Residues within and outside the canonical PBM are important or specific kinase recognition

To functionally confirm these potential associations, we first assessed phosphorylation using HPV-18 E6 as a control. Knowing that the HPV-18 E6 PBM is phosphorylated by multiple kinases, we introduced mutations in key residues of the HPV-66 and HPV-40 PBMs. In the case of HPV-66 E6, we identified critical arginine residues upstream of the canonical PBM, which play vital roles in efficient phosphorylation, and in particular by recognition by PKA. Mutation of the corresponding residues in HPV-18 E6 also confirmed the importance of these amino acids for optimal levels of phosphorylation. In the case of HPV-40 we focused our attention on the carboxy terminal cysteine residue and found that a mutation to a valine is not sufficient for conferring phosphorylation, even though there is an arginine upstream of the PBM. Thus, key residues

within the last 9-10 amino acids of the E6 PBM, both within and outside the canonical PBM play a major role in determining which kinases can phosphorylate E6 and to what degree.

The residues upstream for the PBM, important for phosphorylation, are also critical for binding to PDZ proteins

Using these mutants we then analysed their respective abilities to interact with three well-characterized PDZ domain-containing substrates of HPV-18 E6, hDlg1, MAGI-1 and hScrib. We first confirmed the deleterious effects of having a carboxy terminal cysteine residue in HPV-40 E6 upon interaction with multiple PDZ domain containing proteins, with a valine substitution increasing its interaction with hDlg1, and even more markedly with MAGI-1. In the case of hScrib, the presence of the cysteine affects the GLGF core polar contacts, which are critical for binding. Interestingly, despite the presence of the carboxy terminal valine, this was still not sufficient to confer HPV-40 E6 association with hScrib, indicating that additional critical residues are required for this interaction. These results were also verified by introducing the carboxy terminal valine/cysteine substitution in HPV-18 E6, where this reduced but did not abolish hDlg1 recognition, but completely disrupted recognition of both MAGI-1 and hScrib. Thus, in the case of HPV-40 E6 the carboxy terminal cysteine restricts PDZ target selection and mutating it for a Valine is not sufficient to confer phosphorylation by any kinase, even though the AKT and CHK1 consensus site is present. Indeed, although the presence of a valine or leucine residue in the carboxy-terminal site seems to be high-risk trait, we cannot rule-out that it has a role in target specificity as previously reported (Thomas et al., 2005). In the case of HPV-18 E6, the valine directly interacts with the GLGF motif of the hDlg1-PDZ domain, in a packed manner (Zhang et al., 2007) (Figure 36a). This would correlate with the lower affinity of HPV-16 E6 towards hDlg1 and MAGI-1 , and the fact that HPV-16 E6 is more efficient in degrading hScrib. Considering this, it is likely that substituting the cysteine on HPV-40 with a leucine in p-1 would be more optimal for binding. However, we cannot rule-out that additional residues, might be lacking, that would confer the ability to interact with hScrib and possibly confer phosphorylation by PKA. Using a similar approach with HPV-66, we see that the run of upstream arginine residues is critical, both for optimal levels of phosphorylation by PKA and also for conferring an ability to recognize both MAGI-1 and hScrib. Generating the corresponding mutations within HPV-18 E6 had the similar predicted results, with mutation of the upstream arginine residues greatly decreasing phosphorylation, and at the same time greatly reducing interaction with MAGI-1 and hScrib (Figure 36b). This is in accordance with previously structural report, that shows the presence of a loop connecting the βB and βC strands (BC loop) on the MAGI-1PDZ1 and hDlg1-PDZ3

complexes, which form two similar water-mediated hydrogen bonds, with the arginine in position p-5 of the E6 peptide in both complexes. However, this loop region is poorly conserved among PDZ domains of the same proteins, which might affect the outcome regarding E6 interaction with different PDZ domains of the same protein. Furthermore, our results with the 18E6 R153A mutant, correlate with previously reported results in which a R153G mutant of HPV-18 E6 has reduced ability to induce the degradation of MAGI-1 and hDlg1 (Zhang et al., 2007).

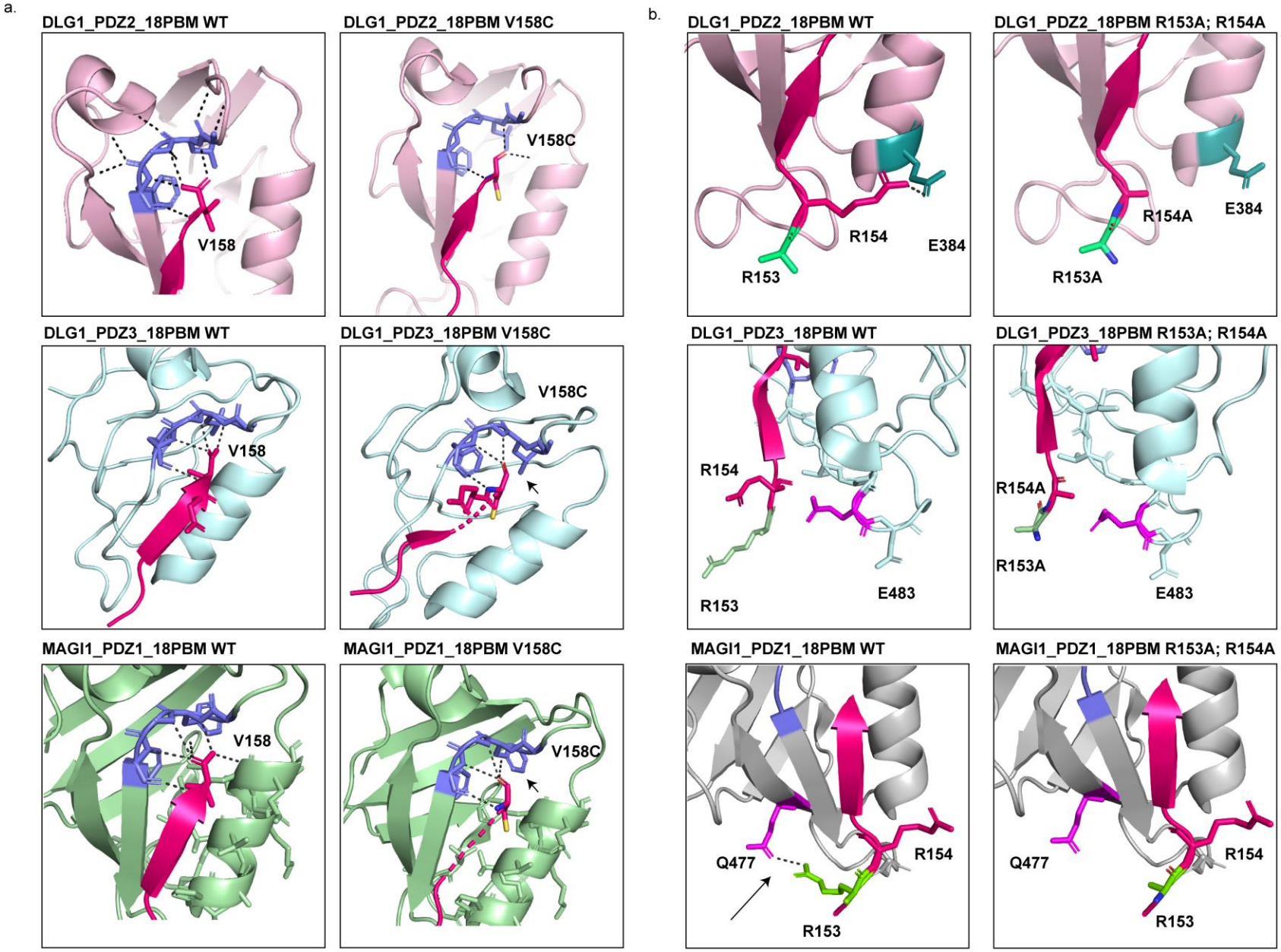

a.
DLG1_PDZ2_18PBM WT
V158
DLG1_PDZ2_18PBM V158C
V158C
DLG1_PDZ3_18PBM WT
V158
DLG1_PDZ3_18PBM V158C
V158C
MAGI1_PDZ1_18PBM WT
V158
MAGI1_PDZ1_18PBM V158C
V158C
b.
DLG1_PDZ2_18PBM WT
R154
E384
R153
DLG1_PDZ2_18PBM R153A; R154A
R154A
E384
R153A
DLG1_PDZ3_18PBM WT
R154
R153
E483
DLG1_PDZ3_18PBM R153A; R154A
R154A
R153A
E483
MAGI1_PDZ1_18PBM WT
Q477
R154
R153
MAGI1_PDZ1_18PBM R153A; R154A
Q477
R154
R153

Figure 36.- Critical residues located in the core and upstream region of the PBM, differentially affect binding to hDlg1 and MAGI-1. (a) The presence of a Cysteine residue in position p-1 on HPV-18 does not completely change conformation and it can also generate polar contacts with most residues of the PDZ hydrophobic pocket; explaining why HPV-40 still can bind hDlg1 and MAGI-1, but with less efficiency in comparison to HPV-18. (b) In the case of R153 in 18PBM, we observed it does not make direct contacts with residues of hDlg1. Conversely, the R154 residue bonds with E384 of the second PDZ domain of hDlg1, explaining why it partially increases binding when introduced into 66PBM. Furthermore, R153 residues makes a critical bond with the Q477 of the first PDZ domain of MAGI-1, explaining why it strongly affects binding.

Taken together, these studies indicate a very close association between the ability of E6 to be phosphorylated within its PBM, and the ability to recognize multiple PDZ domain-containing substrates. We have recently shown that phosphorylation of E6 only occurs in response to particular cellular stresses, with DNA damage resulting in high levels of E6 phosphorylation (Thatte et al., 2018). This suggests that the ability to recognize multiple PDZ substrates is in some way linked to the ability of E6 to be phosphorylated under certain stressed conditions. The conclusion being that in a normal proliferating environment, E6 targeting of multiple PDZ domain-containing proteins offers particular advantages in terms of the viral life cycle. However, when cells are stressed these interactions need to be redirected towards members of the 14-3-3 family of proteins. Indeed a recent publication of the co-crystal structure of 14-3-3σ in complex with 16E6 carboxy terminal wild type peptide, the phosphorylated and the phospho-mimic mutant peptides, have shown the importance of upstream residues for this interaction, since the phosphate group of E6 docks very well the 14-3-3σ protein binding pocket, and is further stabilized by intra-molecular interactions with the arginine residues in p-4 and p-5 (Gogl et al., 2020). Whilst E6-mediated 14-3-3 interactions are implicated with an additional ability to block p53 function, what this has to reveal about the specific functions of the different PDZ targets of E6 remains to be determined. Nonetheless both activities, PDZ and 14-3-3 proteins binding, correlate very closely with high oncogenic potential and both are importantly affected by Arg153.

PART 2

As previously mentioned, HPV-18 and HPV-16 have been shown to cause the majority of cervical and oropharyngeal cancer cases, being HPV-16 the most carcinogenic one (Bruni et al., 2019). Although most of the high-risk E6 proteins are conserved, it is possible to assume that there are specific amino acid sequence determinants which have accumulated and fixed during evolution, on the less conserved carboxy-terminal region, which includes the PBM. Indeed, the linear nature of this region which allows multi-structural protein conformations with multiple PDZ proteins, has aided in the acquisition of sequence mutations, which eventually conferred the high binding promiscuity to HPV-18 and HPV-16 E6 proteins and therefore their carcinogenic potential.

Although the core PBM has a 4-residue consensus sequence, additional residues upstream are also involved in PDZ protein binding, (Hegedüs et al., 2003; Karthikeyan et al., 2001; Kornau et al., 1997; Kornau & Seeburg, 1997; Popovic et al., 2011; Songyang et al., 1997). Indeed, phosphorylation of PBM-bearing proteins typically occurs in position p-2 resulting in negative regulation of PDZ protein binding, but the phosphorylation of upstream serine and threonine residues has also been reported (Hegedüs et al., 2003). A recent study has identified 1926 human PBMs and counted, for each PBM class, the number of potentially phosphorylatable Ser/Thr sites at different positions, as well as the numbers of such sites that have been experimentally proven to be phosphorylated *in cellulo*. They found that up to 82% of all predicted PBMs contain one or more potential phosphorylatable Ser/Thr sites between positions -1 and -10, and that 19% have been found to be phosphorylated at least once on at least one site (Gogl et al., 2020). In the case of HPV E6, *in vitro* phosphorylation by PKA, AKT and CHK1 kinases on position p-2 of the PBM has been reported for most high-risk types, with differences in the phosphorylation efficiency. However, for HPV-31, phosphorylation by PKA on serine residue 82 and on the threonine residue on position p-4 of the PBM have also been shown (Boon et al., 2015), demonstrating that HPV have potential phosphorylatable residue upstream of the PBM.

We have previously focused on upstream residues of the HPV-18 PBM, which are critical for the consensus phosphorylation motif and, at the same time, important for binding PDZ (Boon et al., 2015; Boon & Banks, 2013; Thatte et al., 2018), and 14-3-3 proteins (Gogl et al., 2020). Furthermore, we have shown that there is a correlation between the susceptibility of the E6 protein to be phosphorylated by a given kinase, and the functional flexibility of E6 in binding to PDZ targets (Thomas et al., 2016). Although HPV-18 E6 have been mostly studied, the HPV-16

E6 promiscuity is an additional clear example of the evolving mechanism of the PDZ-PBM interactions directly correlated with carcinogenicity. In this part, we show that HPV-16 E6 upstream region of the PBM can be further post-translationally modified by PKC and CK1 kinases. We believe these modifications are part of a phosphorylation pattern that can achieve a higher level of complexity, which is directly correlated with the carcinogenicity of HPV-16 E6.

Phosphorylation of residues upstream of the PBM of HPV-16 E6 protein can also have an effect on the regulation of PDZ proteins binding

Indeed, we have found a threonine residue (T152) on position p-6 of HPV-16 E6 protein which when phosphorylated can affect the binding to PDZ proteins TIP-1 (Tax1 binding protein 3), SNTB2 (β2-syntrophin) and hScrib. Interestingly, our results show that 16E6 phosphorylated peptides on p-6 bound more TIP-1 protein than the wild type peptides. Likewise, phospho-mimic mutant T152D also bound overexpressed TIP-1 protein more than the wild-type E6. Surprisingly, the T152A mutation had little effect on TIP-1 recognition, as did the ΔPBM mutation. These results demonstrate that TIP-1 recognition by HPV-16 E6 is quite distinct from other PDZ domain containing targets, in that it is not through a classical PBM-PDZ recognition, and furthermore upstream phosphorylation can significantly enhance the level of interaction.

Phosphorylatable residues upstream of the PBM are important for TIP-1 binding

The TIP-1 protein represents a highly unique small protein containing a single PDZ domain, which encompasses residues 12-112 of the 124-amino acid protein (Alewine et al., 2006; Mohanty et al., 2015). It is ubiquitously expressed within multiple organs, being especially rich in epithelia; and it is predominantly localized in the cytoplasm, with rare or undetectable expression in the nucleus or on the cell membrane (Besser et al., 2007; Reynaud et al., 2000; Wang et al., 2010). Generally, TIP-1 works as a tumour suppressor and it is critical in maintaining cell polarity of human epithelial cells (Han et al., 2012a, 2012b, 2013). Furthermore, it probably modulates adhesion, migration and invasion through the β-catenin/cadherin signaling cascade, since it binds the carboxy-terminus of β-catenin protein with high affinity working as a scaffold antagonist and inhibiting its transcriptional activity (Kanamori et al., 2003; Wang et al., 2010; Zhang et al., 2008b). Overall, TIP-1 has been shown to regulate growth both antagonistically and agonistically depending on the specific cancer type. Furthermore, TIP-1 binds the PBM of

protein Kir2.3 and disrupts its localization on the basolateral membrane, causing Kir2.3 to accumulate in an endosomal compartment (Alewine et al., 2006; Yan et al., 2009). TIP-1 also disrupts the PDZ association with the dystrophin-associated protein complex (DPC), the other major basolateral membrane PDZ protein scaffold, in which SNTB2 is included (Alewine et al., 2006; Kachinsky et al., 2000).

The significant conformation changes that TIP-1 adopts in order to accommodate the carboxy-terminus motif of its targets is unusual. It contains a long βB-βC loop region that forms an additional binding site to facilitate peptide binding. Indeed, β-catenin has a PBM which has a highly conserved tryptophan residue in position p-5 (NQLA**W**FDTDL), which forms a hydrogen bond with the Gln43 of TIP-1. This position is highly important since the bulky hydrophobic group of the tryptophan residue is deeply inside the additional binding cavity of TIP-1 and the surrounding alanine (p-6) and phenylalanine (p-4) residues stabilize the cavity (Zhang et al., 2008a).

Besides β-catenin PBM (NQLAWFDTDL), TIP-1 has been shown to bind the glutaminase L protein (KENLESMV) and the Kir 2.3 channel (NISYRRESAI), showing there is not a clear conservation of residues in position p-6, which corroborates the flexibility of TIP-1 protein for binding different PBMs. Furthermore, the TIP-1 'ILGF' motif which substitute the common GLGF motif found in other PDZ proteins have also shown great flexibility in binding (Banerjee et al., 2012; Mohanty et al., 2015; Olalla et al., 2001). Interestingly, modeling of NMR structures, phage display and yeast-two hybrid protein interaction library screening have identified a S/T-X-L/V-D consensus internal binding motif that could also be recognized by TIP-1. In fact, TIP-1 interacts *in vivo* with deltex-1 (DTX1) and Staufen double-stranded RNA binding protein 1 (STAU1), two proteins that lack a carboxy-terminal PDZ domain recognition motif but contain the S/T-X-L/V-D internal recognition motif (Zencir et al., 2013). Structural models of TIP-1 have shown that these internal motifs bind in the pocket of the canonical PBM. Although the binding site for both the internal and carboxy-terminal PBM is the same, the mode of interaction is different. Therefore, the ILGF loop of TIP-1 can easily accommodate these two different binding motifs (Mohanty et al., 2015).

The unique interacting pattern observed with 16E6 wild-type protein and the T152 residue mutants, is explained by the critical role of residues in p-5 and p-6 in the PBM-TIP1-PDZ binding interface. The presence of an extra cavity spanning from p-4 to p-6, suggest that a phosphorylated residue could be better accommodated in the PDZ domain of TIP-1. Furthermore, the flexibility of its PDZ pocket explains why the 16E6 ΔPBM mutant does not abrogate TIP-1 binding as with other PDZ proteins.Because β-catenin binds TIP-1 with high

affinity, it is believed that TIP-1 is a strong competitive inhibitor of other carboxy-terminal β-catenin interacting proteins, including other PDZ domains (Zhang et al., 2008a). It is possible to assume that TIP-1 regulation by E6 has a role in β-catenin stabilization, since the mechanism by which E6/E6AP stabilizes β-catenin is still not clear and the direct interaction of E6 or E6AP with β-catenin has not been proven (Bello et al., 2015; Bonilla-Delgado et al., 2012).

E6-binding to SNTB2 is regulated by residues within and outside the PBM

The other atypical protein we found to bind stronger when threonine residue T152 on position p-6 was phosphorylated was SNTB2 (β2-syntrophin).

β2-syntrophin regulates epithelia cell-cell adhesions. It is also an interactor of Tiam1 protein, and it promotes Tiam1-Rac activity during TJ assembly. Both, GTPase Rac and its activator Tiam1 (T-cell lymphoma invasion and metastasis 1) regulate TJs and AJs and are implicated in tumourigenesis (Mack et al., 2012). Moreover, membrane-localized β2-syntrophin has been documented in normal prostate tissue, and its reduction correlates with prostate cancer progression. It is possible that β2-syntrophin promotes TJ assembly through maintaining correct Tiam-1 localization along the apicobasal axis, in addition to regulating its activity. Overall, the β2-syntrophin-Tiam1-Rac signaling pathway may be deregulated in human prostate cancer (Mack et al., 2012). Additionally, the PDZ-PBM interactions so far described for β2-syntrophin are not conventional. Indeed, Tiam1 contains an internal PBM sequence (KETDI), that when deleted abolishes Tiam1 interaction with β2-syntrophin (Mack et al., 2012). Furthermore, the neuronal nitric oxide synthase (nNOS) associates with β2-syntrophin in muscle cells. The nNOS protein has a β-hairpin finger which acts as a PDZ ligand, docking into the PBM binding groove of the β2-syntrophin PDZ domain. Indeed, the first strand of the nNOS β-finger mimics a canonical PBM. This is evidence that the β2-syntrophin PDZ domain can recognize internal motifs mostly present within a secondary structure that is sterically compatible with the PDZ binding groove (Hillier et al., 1999).

In this case we have observed that the PBM of 16E6 binds β2-syntrophin PDZ domain and residue T156 is crucial for interaction. However, T152 residue is also important for binding, since T152A mutation affects binding and the phospho-mimic mutant T152D increases it. Thus unlike TIP-1, SNTB2 is recognized by E6 in a classical PBM-PDZ interaction, but it retains a common feature in that the association is also stimulated upon upstream phosphorylation of E6 at T152. It is likely that 16E6 binds to the membrane-associated β2-syntrophin, involving itself in the mechanism by which Tiam1-Rac signaling at cell-cell junctions is deregulated (Mack et al.,

2012). Another hypothesis is that E6 binding to β2-syntrophin, is a way of localizing E6 to the basolateral membrane, without affecting β2-syntrophin anchoring.

Even though binding to TIP-1 and SNTB2 is increased, we observed differences between the phosphorylated peptides and the phosphomimic protein pull-down assays. Although phosphorylated amino acids are biochemically different, many *in vitro* and *in cellulo* experiments have involved phosphomimic acidic mutations using glutamine and aspartate residues (Nathan & Yulia., 2013). In fact, drastic differences have been observed between phosphorylated and phosphomimic proteins, the position of the phosphorylated residue and the phosphorylatable residues surrounding the amino acid analysed. Indeed, in the case of HPV-18, the phosphomimic mutant T156E, does not bind 14-3-3σ proteins (Boon & Banks, 2013), probably because an acidic residue within the PBM, which generates a class III PBM, would be more promiscuous, rather than negatively regulating PDZ interactions. In the case of 16E6 PBM the phosphate group of the threonine, mediates several bonds with the PDZ domain, that acidic mutations are unable to make. Furthermore, analysis with the RSK1 kinase PBM, where phosphorylation on p-2 negatively affects interactions, but on p-1 and p-3 mildly modulates them, showed a higher promiscuity in binding by phosphomimic peptides (Gogl et al., 2020; Gógl et al., 2019). Conversely, the phosphomimic peptide of 16E6, mostly decreased the interactions, and hardly generate new unspecific ones, compared to the phosphorylated peptide. We also need to consider the effect of partial phosphorylation in additional phosphorylatable residues upstream of the E6 sequence, which is overcome when using phosphorylated peptides.

Phosphorylation of residues upstream of the PBM can differentially regulate binding, depending on the target PDZ protein

Moreover, conversely of what is observed with TIP-1 and SNTB2 proteins, phosphorylation of E6 in position p-6, decreases the interaction with ZO-1 protein. Indeed, regulation of phosphorylation in positions upstream of the PBM has been extensively characterized, specifically with Claudins and ZO protein interactions. Most Claudins share a PBM at the extreme carboxyl terminus which mediates binding to ZO-1, ZO-2, and ZO-3 proteins (Itoh et al., 1999). In the case of claudin-1 and the PDZ1 of ZO-1 domain, residues beyond p-3 are not observed to make any direct interaction with the PDZ1 domain. But for Claudin-2 and Claudin-16, the residues important for interaction span until position p-6, which has a tyrosine that intercalates in the B2:B3 loop, stabilizing the hydrophobic interactions. In this case, phosphomimic mutants considerably lower the affinity to PDZ1, and introducing this tyrosine

residue into Claudin-1 enhances ZO-1binding. This explains the lack of binding to, specifically ZO-1, when we mutated to a phospho-mimic residue on position p-6. The residue in p-6, is critical for creating high affinity binding. The threonine residue in p-6 in E6 has a similar effect on binding than tyrosine, and the phosphorylation state in this position might be a physiological mechanism for negatively modulate the affinity for ZO-1 (Lee & Zheng, 2010b; Van Itallie et al., 2012).

Likewise HPV E6, the cytoplasmatic tails are the less conserved region of claudins and are enriched in phosphorylatable residues (Van Itallie & Anderson, 2018). Several studies have demonstrated phosphorylation-induced regulation of claudins by PKC (D'Souza et al., 2007); PKA (D'Souza et al., 2005) and WNK4 kinases (Ohta et al., 2006). In some cases, it has been observed that phosphorylation affects the ability to claudins (Claudin-16) to interact with ZO-1, as we observed with phosphorylated 16E6 (Popovic et al., 2012). Furthermore, Claudins can be phosphorylated in residues far upstream of the PBM. Claudin-2 is multiply phosphorylated on serine, threonine, and tyrosine residues on the cytoplasmic carboxy-terminal domain, with a major phosphorylation site identified on Ser208. The phospho-mimic mutants of Ser208 localized to cell-cell contacts better than the non-phosphorylatable mutant. Possibly, the cell membrane localization was required for phosphorylation at Ser208, however, phosphorylation could also be required for membrane targeting. In any case, Ser208 phosphorylation is not an absolute requirement for membrane localization, since the non-phosphorylatable S208A could still localize to the membrane although much less efficiently (Van Itallie et al., 2012).

Although phosphorylation at position -2 has the most drastic effect in binding, because mainly it disrupts the β conformation of the bound E6 peptide, here we observed that phosphorylation of 16E6 at lower-impact sites tends to preserve binding, while modulating affinity and specificity. This correlates with previous studies with the PBM of the β2AR, which harbors three phosphorylatable residues, and have shown that the native PBM binds moderately to the PDZ domain of SNX27, whereas its phosphorylated variants at position p-5 and p-6 bind more strongly, and the variant phosphorylated at p-2 binds weakly (Clairfeuille et al., 2016).

HPV-16 E6 can be singly and multiply phosphorylated outside the PBM by PKC and CK1

To further identify the kinase responsible for phosphorylating T152, we analyzed the amino acid sequence of HPV-16 E6 with the prediction software NetPhos3.1 and obtained a high score for PKC, not only for threonine p-6, but for three serine residues (S154; S149; S150) upstream of the PBM.

While doing *in vitro* phosphorylation assays, we found that PKC does not phosphorylate T152. However, CK1 kinase, which does not have the consensus phospho-acceptor site and had an equivalent score for all possible phosphorylatable residues on E6, was responsible for partially phosphorylate T152 and the PBM. Importantly, we found that both kinases were responsible for a multiple phosphorylation event in three upstream serine residues (S154; S149; S150); and that subsequently mutating the most upstream S145 or the doublet S149; S150, to alanine residues, does not cause the same drastic effect on phosphorylation as substituting the three serine residues at the same time. Interestingly, this highly phosphorylated state of E6, does not seem to affect its oncogenic function of degrading p53, which was expected since the minimal reported segment still functional for p53 degradation comprises residues 1-142 (E6 Δ143-151) (Lipari et al., 2001; Liu et al., 2009; Martinez-Zapien et al., 2016). Since a multiple aspartate substitution could affect the carboxy-terminal structure, we assessed the ability of mutant E6 to bind common PDZ targets: hScrib and Dlg1. We found that binding is not abrogated, but mildly altered, therefore we assumed that the functionality of E6 is not affected by these substitutions. We also found that the multiple phosphorylation event in E6 protein changes its localization, showing more retention in the cytoplasm when the three serine residues are substituted by aspartate residues. Conversely, higher retention in the nucleus was observed with the corresponding alanine substitution mutants.

HPV-16 E6 PBM is also phosphorylated, which might represent an initial step in the recruitment of E6 to the membrane and therefore a key element in promoting the phosphorylation of the triple serine residues, which have shown to be a conserved mechanism of HPV-16 E6.

Phosphorylation by PKC has been previously associated with cellular distribution. The PKC branch of ACG kinase family tree has 10 family members that are not only regulated by phosphorylation but also by interaction with binding partners (O'Neill et al., 2011). From these members, PKCα is a scaffold protein of the cytoplasmic signaling and scaffolding Par complex (Par3-Par6-PKC), which is associated with Adherent Junctions (AJ) and Tight Junctions (TJ) and regulates junction assembly and polarity; having a direct involvement with cancer cell survival and migration (Larsson, 2006; O'Neill et al., 2011).

We hypothesized that PKCα was the most suitable PKC isoform for phosphorylating E6, since it serves a particularly important function in signaling in the polarity complex (Rosse et al., 2010). Furthermore, similar to high-risk E6 proteins, only PKCα contains a class I PBM (QSAV), capable of interacting with the PDZ-scaffold protein PICK1 (protein that interacts with C kinase 1) (Lee & Zheng, 2010b; Staudinger et al., 1997), Dlg4 and Dlg1 (Colledge et al., 2000; Lim et al., 2002); suggesting that PDZ interactions are important to coordinate the signaling of PKCα in the cellular

membrane. Additionally, PKCα PBM interacts with the third PDZ domain-containing of DLG1 and phosphorylates it on T656. Importantly, Dlg1 phosphorylation by PKCα induced its translocation and further co-localization with PKCα at the leading edge of migrating cell. This interaction was required for PKCα to promote cellular migration, probably by Dlg1 acting as a scaffold for PKCα substrates and to control PKC-dependent regulation of cellular migration (O'Neill et al., 2011). Moreover, PKCα phosphorylation of the PDZ domain of PICK1 on S77, also affects its cellular distribution (Ammendrup-Johnsen et al., 2012). Different from PKCα effect on Dlg1, the PKCα-phosphorylation of PICK is independent of the interaction of PKCα PBM with the PICK's PDZ domain.

Likewise PKC, CK1 was also found to multiply phosphorylate the 16E6 protein with a possible outcome in E6 cellular distribution. The CK1 family members are constitutively active in many different tissues and cell lines. Moreover, several effectors like cellular stress can modulate CK1 expression and result in elevated levels of CK1 activity and protein expression (Tuazon & Traugh, 1991; Xu et al., 2010). All CK1 isoforms are biochemically related but have different physiological roles in cells. Particularly, the CK1α isoform is responsible for the hyperphosphorylation of the NS5A protein from HCV. The NS5A phosphorylation center is made of eight highly conserved serine residues in the LCS I region of NS5A, from where three tandem serine phosphorylation sites S222, 235 and 238 have been identified. CK1α kinase preferentially phosphorylates serine or threonine residues when the upstream -3 position is phosphorylated and it directly phosphorylates S225, S232 and S235, and possibly other serine residues upstream. The outcome of this multiple phosphorylation event on NS5A depends on the HCV genotype and it is mostly related to the enhancement of replication, probably via enhancing the interaction with hVAP-A (Chong et al., 2016).

Furthermore, the CK1ε isoform, have been implicated in the Wnt/ β-catenin pathway. Without the Wnt stimuli, CK1ε phosphorylates β-catenin at S45, providing a priming site for the subsequent GSK3β phosphorylation on T41, S37 and S33 residues (Liu et al., 2002). β-catenin, gets phosphorylated by GSK3β at multiple S/T residues, which is then recognized by B-TrCP, polyubiquitinated and thus targeted for proteasomal degradation (Zhou & Hung, 2005). When Wnt stimulation occurs, the GSK3β activity towards β-catenin is inhibited, inducing the accumulation of the protein in cells (Jong et al., 2005; Yook et al., 2006). A similar event has been reported for Snail, a Zn-finger transcription factor associated with the expression of E-cadherin, in which CK1ε is required for the Snail degradation mediated by phosphorylation (Batlle et al., 2000). Likewise, S104 and S107 residues, located in a proline-rich sequence, which is not a canonical target sequence of CK1ε, are key in the phosphorylation of Snail by CK1ε and

GSK3β and the cellular stability of Snail (Xu et al., 2010). As with Snail, mutating the serine residues to alanine in 16E6 increases its stability and the retention in the nucleus. Whether the cellular distribution observed in the E6 serine/alanine mutants could be partly a mechanism of degradation induced by phosphorylation of serine residues, needs to be further investigated.

Although CK1 is considered a priming kinase and GSK3 a primed kinase, CK1 can also perform non-primed phosphorylation, dictated by non-canonical consensus sequences, whose specificity determinants are not defined, but conjectural. Owing to its ability to perform hierarchical phosphorylation in opposite directions and with different spacing between phosphoresidues, CK1 phosphorylation of E6 could be triggered by the phosphorylation of one or more amino-terminal residues by AKT, PKA, PKC or other basophilic kinases, adding to the number of functional states of E6. Once phosphorylated, these residues could start a downstream cascade of phosphorylation events catalyzed by CK1, affecting all serine/threonine residues located at n+3 positions relative to those already phosphorylated until the carboxy terminus is reached. Furthermore, we cannot discard that phosphorylation in the PBM, also starts upstream sequential events of phosphorylation by CK1, or other kinases.

Many oncogenes and onco-suppressors encode proteins equipped with serine clusters which may be instrumental to their functional plasticity. From all PTMs, phosphorylation is the most abundant modification type. Three quarters of the human proteome is composed of phosphoproteins. In fact, singly phosphorylated proteins were once believed to be the rule, and they are now the exception, since the large majority of phosphoproteins are being multi-phosphorylated. This represents a way by which distinct signaling pathways can crosstalk with each other (Cesaro & Pinna, 2015). Furthermore, there is a correlation between phosphorylation and protein localization, which is regulated by the PTM interplay. While acetylation of the nuclear localization signal (NLS) serves as a signal for nuclear import, phosphorylation is a signal for cytoplasmic retention (Harrison et al., 2010), and SUMOylation has also been reported as a signal to initiate the transport to the nucleus (Minguez et al., 2012).

Multiple phosphorylation would not be a new mechanism in HPV. Since several of its proteins have been previously shown to be highly post-translationally modified on serine and threonine residues. A very well-studied example is the HPV E4/E1 protein which gets highly phosphorylated in multiple sites during cell-cycle stages and differentiation, with outcomes in localization and protein structure (McIntosh et al., 2010). Overall, the regulation of the E1/E4 function by the specific kinases is an example of different states of phosphorylation driven by individual epithelial tropism and cell cycle phases (Deng et al., 2004; Doorbar, 2013; Ma et al., 1999; Yu et al., 2007), through which E6 protein also goes through.

PART 3

The majority of human papillomaviruses are low-risk types, from which the alpha low-risk types HPV-11 and HPV-6 cause benign hyper proliferative lesions such as genital warts or condylomata acuminata (CA), becoming one of the most widespread sexually transmitted diseases , not frequently associated with malignant carcinoma among the general population (Bruni et al., 2019; Egawa & Doorbar, 2017; Jamshidi et al., 2012; Sterling et al., 2001). However, low-risk HPV infections have been quite rarely detected in invasive cervical and anal cancer (Cornall et al., 2013; Guimerà et al., 2013; Li et al., 2011). Particularly, there are HPV-11-positive cases of cervical and anal squamous cell carcinomas, malignantly transformed laryngeal papillomas and sinonasal inverted papillomas associated with squamous cell carcinoma . Furthermore, HPV-11 as well as other low-risk types are involved in cancer development in immunosuppressed individuals and in certain genetic backgrounds (RPP), in which the progression route towards cancer is different . In cases of persistent laryngeal papillomas, it has been observed that the viral genome integrates into the host cell chromosome, suggesting deregulated viral gene expression . This has also been described for Beta HPV types, which are potential significant cause of non-melanoma skin cancer , in which deregulation of viral gene expression is required and it is not tolerated by immunosuppressed or EV background hosts .

Moreover, the low-risk HPVs have been as successful as high-risk types in molecular evolution and niche adaptation, their life cycles share similar strategies for productive infection, but high-risk types have evolved functions which have conferred them oncogenic properties. Indeed, low risk types are maintained and propagated in the general population as successfully as the oncogenic high-risk types (De Koning et al., 2015; De Koning et al., 2007). They infect long-lived basal epithelial cells, including stem or stem-like cells (Egawa et al., 2003; Egawa et al., 2015; Herfs et al., 2012), and their E6 and E7 proteins can modify these cells, limiting their differentiation and maintaining stem-like characteristics to stablish a productive infection. Conversely to high risk types, the low-risk E6 and E7 proteins do not drive extensive cell proliferation in these cells, since they have a more restrict transcriptional regulation (Egawa & Doorbar, 2017; Gheit, 2019). Furthermore, it is believed that a major function of low-risk E6 is to inhibit apoptosis via Bak degradation and prevent differentiation (Thomas & Banks, 1999; Underbrink et al., 2016), more than inhibiting apoptosis through p53 degradation. Moreover, HPV-11 E6 associates with E6AP in vivo , forming the low-risk HPV E6-E6AP complex, needed for the degradation of Bak (Thomas & Banks, 1999) and NHERF-1 (Drews et al., 2019). This suggest HPV-11 E6 could possibly target other E6-associated proteins for degradation, however no experimental information have confirmed this.

HPV-11 E6 can degrade p53 depending on its phospho-status

Importantly, low-risk E6 proteins weakly bind p53 through a region within the carboxy-terminal half of E6 that is conserved among alpha types . In the case of high-risk E6 proteins, besides binding to the carboxy-terminal site, they also bind to the p53 core domain which is necessary for p53 E6AP-depended degradation, and which low-risk E6s do not bind to . Other studies have also suggested that a second region within the amino-terminus of p53 is required for degradation, but this region is also crucial for the native conformation of p53 and therefore, limited binding to E6 was most probably associated with poor conformation of p53 (Li & Coffino, 1996; Mansur et al., 1995).

Considering degradation of p53 by 11E6 has recently been observed (Murakami et al., 2019), we aimed to assess the molecular basis for this and reasoned that it could be modulated by the DNA damage response as this is one of the major ways in which p53 is regulated, and which has been shown previously to enhance p53 degradation by the high risk HPV E6 oncoproteins. Indeed, we show that 11E6 degrades p53 in conditions of high confluence and DNA damage induction, which corroborates previous studies showing that degradation of p53 by 11E6 is observed depending on cell density and the levels of viral gene expression. Indeed, Muramaki et al, showed that 11E6 was necessary in order to sustain low-risk HPV genome copy number as cell confluence was reached, and similarly to 16E6, this was dependent on E6-mediated p53 proteasomal degradation, allowing E6 proteins to support genome amplification, while limiting the extent of E6-mediated cell proliferation (Murakami et al., 2019).

To further confirm the effect of DNA damage in p53-degradation by 11E6, we used Etoposide, which is a widely used drug for chemotherapy that induces DNA damage by inhibition of Topoisomerase II (Burden et al., 1996). Etoposide-treated cells accumulate at G2/M, which can occur in both p53-dependent and p53-independet manner (Clifford et al., 2003). We observed that 11E6 can degrade p53 in the presence of the Etoposide. Although this is an exogenous stimulus, the DNA damage response is activated in HPV infection. Indeed, E6 alters the cellular environment to allow long-term maintenance of extrachromosomal elements, and the presence of extrachromosomal DNA in normal cells is not tolerated and is likely sensed as a DNA damage stimulus. Furthermore, it has been reported that high-risk types induce phosphorylation of p53, probably through induction of the DNA damage response (Oh et al., 2004).

During the DNA damage response, ATM and ATR kinases, induce and activate p53 by phosphorylation, with differences in the level of phosphorylation, depending in the stress

inducing agent. Indeed, Etoposide induces the phosphorylation of p53 on Ser15 by different kinases (Gao et al., 1999), which has been considered a nucleating event in p53 activation, that promotes the sequential modification of many subsequent residues (Saito et al., 2003; Saito et al., 2002; Sakaguchi et al., 1998, 2000). We corroborated that the active form of p53 phosphorylated in Ser15 was the target for 11E6 degradation by using the phosphomimic mutant of p53 S15D. We performed half-life experiments, collecting the samples every 30 minutes over a period of 2 hours, and consistently observed degradation of p53 S15D, but not of the alanine substitution mutant S15A. Furthermore, this correlated with the levels of wild type p53 degradation induced by 11E6, following Etoposide treatment. Although we have not identified the kinase responsible for phosphorylating p53 at S15 in these assays, or the pathway involved, we believe that ATM and ATR are most likely involved, since both provide the continuity of Ser15 phosphorylation for several hours after the initial DNA damage induced stimulus. However, other kinases like DNA-activated protein kinase (DNA-PK) could also phosphorylate p53 in vitro on Ser15 and Ser37 and after Etoposide stimulus (Thompson et al., 2004).

HPV-11 E6 colocalizes with the phosphorylated form of p53, mostly in the cytoplasm of the cell

We further confirm that 11E6 was targeting the active form of p53 by immunofluorescence. 11E6 colocalized with wild type p53 after Etoposide treatment, and similarly, it colocalized with the S15D mutant in the cytoplasm. Moreover, previously reported studies have also shown that 11E6 localizes in the cytoplasm, and that this could altered the subcellular localization of p53 (Sun et al., 2008). Considering this, it is possible to suggest that following transfection of 11E6, the p53 carboxy-terminal NLS is masked, interfering with nuclear import of p53. Indeed, the ability of p53 to localize to the nucleus is essential for p53 to act as a transcription factor, and modification of its localization represents an important regulatory mechanism. Furthermore, 11E6 binding to the carboxy terminal region of p53 have been shown to inhibit the p53-mediated repression of transcription (Lechner et al., 1992), possibly through blocking of the p53 oligomerization domain and the signal for nuclear localization, located on the carboxy-terminus of p53. Moreover, we also observed more cytoplasmatic distribution of the wild type p53 and the S15D mutant in the presence of 11E6, conversely with what has been reported, that Ser15 phosphorylation masks a nuclear export signal, contributing to the retention of p53 within the nucleus (Zhang & Xiong, 2001). Additionally, the HTLV-1 Tax oncogenic protein can also interact with phosphorylated forms of p53, but in this case leading to stabilization and transcription inactivation of wild-type p53 (Pise-Masison et al., 1998).

Whilst several studies have reported the potential for low risk HPV E6 proteins to interact with E6AP, bind p53 and degrade it under certain circumstances the molecular basis for this has remained obscure. These studies begin to provide a mechanistic basis for how low risk HPV-11 E6 can promote degradation of p53, and this is linked directly to the DNA damage response within the infected cell and the subsequent activation of p53. Indeed this had been reported previously for high risk HPV E6 proteins, where p53 appears more susceptible to degradation following induction of DNA damage (Kessis et al., 1993; Mantovani & Banks, 1999). Here we show that phosphorylation of p53 at S15 enhances its susceptibility to 11E6 induced degradation. However, whether the p53 degradation observed is induced directly by enhanced 11E6 binding to phospho-p53 on Ser15 needs to be investigated. Furthermore, since Ser15 is located in the amino-terminal site, and its DNA-damage induced phosphorylation recruits several proteins in order to stabilize p53, we hypothesize that E6 could still be binding the carboxy-terminal site of the active form, but upon the DNA damage stimuli, it would be blocking the binding of p53-stabilizing proteins, which bind or post-translationally modify the carboxy-terminal site in order to stabilize p53. Indeed, previous studies have reported that HPV-11 can inhibit the p53 transactivation activity in an E6AP-dependent manner through association with p300 acetyltransferase/transcriptional co-activator (Crook et al., 1991; Thomas & Chiang, 2005), which acetylates p53, after binding to the phosphorylated amino-terminal site of p53.

Implications of 11E6-mediated degradation of phospho-p53 in HPV evolution

It is also interesting to speculate on the role of 11E6 degradation of phospho-p53 on Ser15, in the context of evolution. It is possible to suggest that specific activated forms of p53 might have been a common target of the ancestor of the low-risk and high-risk types. This virus was able to degrade specific forms of p53 to favor genome maintenance and replication and probably overcome differentiation of the infected cell. Eventually during the evolution of the virus, the ancestors of the high-risk types developed the ability to degrade all form of p53, allowing the carcinogenic process to happen. This is supported by the idea that high-risk HPV types-induced carcinogenesis is not an evolutionary advantage for the virus since it eventually kills the infected host. In support of this, Ser15 phosphorylation has a broader role in p53 activation, since basal levels of p53 and p53 induced by Mdm2-inhibition show detectable levels of Ser15 phosphorylation . Experiments with the alanine substitution of Ser15, unable to be phosphorylated, impairs the growth arrest induced by p53. Conversely, the phosphomimic mutations to aspartate, maintains the p53 transcriptional and biological functions. Furthermore, the ability of p53 to be loaded on promoters such as p21, ready to start transcription following a given stimulus, is impaired when Ser15 is mutated to

alanine . Additionally, Ser15 is also phosphorylated through the AMPK pathway in response to glucose depletion and mediates p53-dependent metabolic arrest at G1/S, suggesting ser15 has a critical role for cellular stresses that are independent of DNA damage (Jones et al., 2005). Furthermore, phosphorylation on Ser15 peaks during G1 , suggesting that the p53-mediated grow arrest in G1 could be overcame by 11E6 through degradation of p53 activated forms.

Likewise, we cannot rule out the effect of p53 degradation by low-risk E6 in differentiation. This is more clear in the case of E6 proteins from beta-HPV types, since instead of binding E6AP, they bind MAML, a downstream component of the Notch signaling pathway, which controls the normal basal cell differentiation (Brimer et al., 2017; Meyers et al., 2013). Furthermore, there is a link between the HPV genome replication and the inhibition of Notch signaling. It is suspected that the use of Notch pathway may reflect the different roles of p53 in regulating differentiation according to epithelial sites, and the adaptation of HPVs to different epithelial niches where the role of p53 is more or less important (Kranjec et al., 2017; Murakami et al., 2019). Experiments in NIKS cells, have shown that although HPV11 E6 and E7 failed to significantly increase the levels of cell-cycle entry of NIKS or to promote the degradation of p53 at sub-confluence; in post-confluent cells, HPV11 E6 leads to loss of p53 and inhibition to differentiation. Interestingly, the 11E6 mutants (W113R), which cannot bind to E6AP (Brimer et al., 2007; Oh et al., 2004), still reduced p53 levels at post-confluence, suggesting that 11E6 mediates p53 degradation in an E6AP-independent manner. Although caution is required in excluding E6AP, as siRNA ablation would provide more compelling evidence that the effects are E6AP independent. Therefore, HPV11 E6 is able to exploit the proteasomal pathway in order to target p53 for degradation, and probably this mechanism is regulated in the epithelial basal layer to support virus genome maintenance in response to increasing cell density and the eventual need to differentiate and enter the virus productive cycle.

Further experiments need to be performed in the context of stable 11E6 protein and endogenous p53. It would be important to stablish if degradation of the active form of p53 is a newly acquired trait of HPV-11 that we were able to observe during the induction of DNA damage response, or whether it is mostly an ancestral mechanism of all alpha HPV types in order to maintain replication and avoid differentiation.

APPENDIX I

Proteomic Analysis of HPV-16 biotinylated peptides by Mass spectrometry

Peptide: SCRAMBLE (SCR)															
rank	log(e)	log(I)	% (measured)	% (corrected)	unique	total	Mr	Identifier	Accession	Description	Chromosome	Start	End	Strand	Band
1	-144.2	7.46	19	30	13	29	82.9	HADHA	ENSP00000370023	HADHA:p , hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha [Source:HGNC Symbol;Acc: HGNC:4 ...	2	26413504	2.6E+07	-1	p23.3
2	-139	7.29	17	28	12	22	66	KRT1	ENSP00000252244	KRT1:p , keratin 1 [Source:HGNC Symbol;Acc: HGNC:6412]	12	53068520	5.3E+07	-1	q13.13
3	-112.5	7.25	18	32	7	23	46.5	LUC7L2	ENSP00000347005	LUC7L2:p , LUC7 like 2, pre-mRNA splicing factor [Source:HGNC Symbol;Acc: HGNC:21608]	7	139025105	1.4E+08	1	q34
4	-100	6.94	15	23	8	12	62	KRT9	ENSP00000246662	KRT9:p , keratin 9 [Source:HGNC Symbol;Acc: HGNC:6447]	17	39722096	4E+07	-1	q21.2
5	-95.5	7.27	17	26	9	19	51.3	HADHB	ENSP00000325136	HADHB:p ,	2	26466038	2.7E+07	1	p23.3
6	-58	6.64	6.6	11	4	6	62.3	KRT5	ENSP00000252242	KRT5:p , keratin 5, type II [Source:HGNC Symbol;Acc: HGNC:6442]	12	52908359	5.3E+07	-1	q13.13
7	-51.5	6.79	4.3	7	1	1	38.4	LUC7L	ENSP00000337507	LUC7L:p , LUC7 like [Source:HGNC Symbol;Acc: HGNC:6723]	16	238968	279462	-1	p13.3
8	-38.7	6.57	12	14	4	6	41.7	ACTB	ENSP00000349960	ACTB:p , actin beta [Source:HGNC Symbol;Acc: HGNC:132]	7	5566782	5603415	-1	p22.1
9	-37.5	6.55	15	30	4	6	43.4	PTRF	ENSP00000349541	PTRF:p ,	17	40554470	4.1E+07	-1	q21.2
10	-35.2	6.25	9.4	18	4	4	59.3	RBM39	ENSP00000253363	RBM39:p , RNA binding motif protein 39 [Source:HGNC Symbol;Acc: HGNC:15923]	20	34291531	3.4E+07	-1	q11.22
11	-31.4	6.51	7.4	9	4	5	58.9	AHCYL1	ENSP00000358814	AHCYL1:p ,	1	110527308	1.1E+08	1	p13.3
12	-31.3	6.87	2	3	1	1	65.4	KRT2	ENSP00000310861	KRT2:p , keratin 2 [Source:HGNC Symbol;Acc: HGNC:6439]	12	53038342	5.3E+07	-1	q13.13
13	-31.3	6.28	10	16	3	4	27.9	U2AF1	ENSP00000291552	U2AF1:p , U2 small nuclear RNA auxiliary factor 1 [Source:HGNC Symbol;Acc: HGNC:12453]	21	44513066	4.5E+07	-1	q22.3
14	-31	6.67	5.1	8	4	7	76.6	NCL	ENSP00000318195	NCL:p , nucleolin [Source:HGNC Symbol;Acc: HGNC:7667]	2	232318242	2.3E+08	-1	q37.1
15	-29.7	6.49	5	9	3	7	58.8	KRT10	ENSP00000269576	KRT10:p , keratin 10 [Source:HGNC Symbol;Acc: HGNC:6413]	17	38974369	3.9E+07	-1	q21.2
16	-29.5	7.15	24	30	3	9	14.4	SUB1	ENSP00000422078	SUB1:p , SUB1 homolog, transcriptional regulator [Source:HGNC Symbol;Acc: HGNC:19985]	5	32531739	3.3E+07	1	p13.3
17	-25.7	6.39	8.6	12	3	5	47.7	SQSTM1	ENSP00000374455	SQSTM1:p ,	5	179233388	1.8E+08	1	q35.3
18	-21.2	5.36	7.4	11	1	1	27.3	NUCKS1	ENSP00000356110	NUCKS1:p , nuclear casein kinase and cyclin dependent kinase substrate 1 [Source:HGNC Symbol;Acc: HGNC:29923]	1	205681947	2.1E+08	-1	q32.1
19	-21.1	6.18	4.7	6	3	3	60.5	CDC73	ENSP00000356405	CDC73:p ,	1	193091147	1.9E+08	1	q31.2
20	-20.1	6.78	17	29	2	4	14.8	RPL22	ENSP00000346088	RPL22:p , ribosomal protein L22 [Source:HGNC Symbol;Acc: HGNC:10315]	1	6241329	6269449	-1	p36.31
21	-20	6.8	10	22	2	8	25.5	SRSF2	ENSP00000376276	SRSF2:p , serine and arginine rich splicing factor 2 [Source:HGNC Symbol;Acc: HGNC:10783]	17	74730197	7.5E+07	-1	q25.1
22	-19.7	6.04	8	12	3	3	50.1	TUBA1B	ENSP00000336799	TUBA1B:p , tubulin alpha 1b [Source:HGNC Symbol;Acc: HGNC:18809]	12	49521565	5E+07	-1	q13.12
23	-16.7	5.22	4.9	6	1	1	41.6	EIF3H	ENSP00000276682	EIF3H:p ,	8	117654369	1.2E+08	-1	q24.11

24	-15.7	6.56	12	23	2	5	19.3	SRSF3	ENSP00000362820	SRSF3:p , serine and arginine rich splicing factor 3 [Source:HGNC Symbol;Acc: HGNC:10785]	6	36562145	3.7E+07	1	p21.31
25	-14.9	6.41	2.5	3	1	1	51.5	KRT14	ENSP00000167586	KRT14:p , keratin 14, type I [Source:HGNC Symbol;Acc: HGNC:6416]	17	39738531	4E+07	-1	q21.2
26	-14.5	6.22	16	28	2	4	23.7	ENSP00000441406	ENSP00000441406	ADP ribosylation factor like GTPase 6 interacting protein 4 [Source:HGNC Symbol;Acc: HGNC:18076]	12	123464333	1.2E+08	1	q24.31
27	-14.5	6.66	18	27	2	3	16.8	CALM2	ENSP00000272298	CALM2:p ,	2	47272677	4.7E+07	-1	p21
28	-13.4	5.82	14	21	2	2	17.7	RPS18	ENSP00000393241	RPS18:p , ribosomal protein S18 [Source:HGNC Symbol;Acc: HGNC:10401]	6	33239787	3.3E+07	1	p21.32
29	-13	5.9	1.3	2	2	3	144.3	TCOF1	ENSP00000409944	TCOF1:p , Treacher Collins-Franceschetti syndrome 1 [Source: HGNC 11654]	5	149737202	1.5E+08	1	q32
30	-12.5	6	4.1	8	2	2	50.1	EEF1A1	ENSP00000339063	EEF1A1:p , eukaryotic translation elongation factor 1 alpha 1 [Source:HGNC Symbol;Acc: HGNC:3189]	6	74225473	7.4E+07	-1	q13
31	-12.4	6.03	19	22	2	3	11.7	TXN	ENSP00000363641	TXN:p , thioredoxin [Source:HGNC Symbol;Acc: HGNC:12435]	9	113006091	1.1E+08	-1	q31.3
32	-12.1	6.03	1.9	3	2	2	117.4	DDX46	ENSP00000416534	DDX46:p ,	5	134094469	1.3E+08	1	q31.1
33	-11.9	6.29	12	13	2	3	20.5	CAV1	ENSP00000339191	CAV1:p ,	7	116164839	1.2E+08	1	q31.2
34	-11.1	6.05	6.8	9	2	2	32.6	NPM1	ENSP00000428755	NPM1:p , nucleophosmin (nucleolar phosphoprotein B23, numatrin) [Source:HGNC Symbol;Acc: HGNC:7910]	5	170814120	1.7E+08	1	q35.1
35	-10.8	6.16	36	52	2	2	10.9	C19orf33	ENSP00000301246	C19orf33:p , chromosome 19 open reading frame 33 [Source:HGNC Symbol;Acc: HGNC:16668]	19	38794801	3.9E+07	1	q13.2
36	-10.2	5.63	4.5	7	2	2	49.6	TUBB	ENSP00000339001	TUBB:p , tubulin beta class I [Source:HGNC Symbol;Acc: HGNC:20778]	6	30687978	3.1E+07	1	p21.33
37	-8.4	5.46	2.4	6	1	1	59.2	CPSF6	ENSP00000391774	CPSF6:p ,	12	69633317	7E+07	1	q15
38	-7.5	6.09	3.9	6	1	2	28	RPL8	ENSP00000378378	RPL8:p , ribosomal protein L8 [Source:HGNC Symbol;Acc: HGNC:10368]	8	146015150	1.5E+08	-1	q24.3
39	-7	6.03	2.3	4	1	3	47.7	RPL4	ENSP00000311430	RPL4:p , ribosomal protein L4 [Source:HGNC Symbol;Acc: HGNC:10353]	15	66790355	6.7E+07	-1	q22.31
40	-6.6	6.08	3.1	5	1	2	40.2	RPL3	ENSP00000386101	RPL3:p , ribosomal protein L3 [Source:HGNC Symbol;Acc: HGNC:10332]	22	39708887	4E+07	-1	q13.1
41	-5.7	5.42	3.1	5	1	1	56	RCC2	ENSP00000364585	RCC2:p ,	1	17733256	1.8E+07	-1	p36.13
42	-5.5	6.13	4.7	9	1	3	21.8	HIST1H1A	ENSP00000244573	HIST1H1A:p , histone cluster 1 H1 family member a [Source:HGNC Symbol;Acc: HGNC:4715]	6	26017260	2.6E+07	-1	p22.2
43	-5.3	5.43	6.2	9	1	1	16.1	RPS19	ENSP00000221975	RPS19:p , ribosomal protein S19 [Source:HGNC Symbol;Acc: HGNC:10402]	19	42363988	4.2E+07	1	q13.2
44	-5.1	5.23	3.2	4	1	1	34.3	RPLP0	ENSP00000376299	RPLP0:p , ribosomal protein lateral stalk subunit P0 [Source:HGNC Symbol;Acc: HGNC:10371]	12	120634489	1.2E+08	-1	q24.23
45	-4.3	5.4	2.2	4	1	1	57.2	PPIL4	ENSP00000253329	PPIL4:p ,	6	149825869	1.5E+08	-1	q25.1
46	-4.2	6.14	2.6	3	1	1	74.8	GIMAP8	ENSP00000305107	GIMAP8:p , GTPase, IMAP family member 8 [Source:HGNC Symbol;Acc: HGNC:21792]	7	150147718	1.5E+08	1	q36.1
47	-4.1	5.94	5.1	11	1	1	23.4	RPL14	ENSP00000345156	RPL14:p , ribosomal protein L14 [Source:HGNC Symbol;Acc: HGNC:10305]	3	40498783	4.1E+07	1	p22.1
48	-4	5.56	4.1	7	1	1	32.8	RPSA	ENSP00000346067	RPSA:p , ribosomal protein SA [Source:HGNC Symbol;Acc: HGNC:6502]	3	39448180	3.9E+07	1	p22.1
Peptide: HPV-16 PBM wild-type (WT)															
rank	log(e)	log(I)	% (measured)	% (corrected)	unique	total	Mr	Identifier	Accession	Description	Chromosome	Start	End	Strand	Band
1	-392	7.91	25	34	34	60	174.8	SCRIB	ENSP00000322938	SCRIB:p , scribbled planar cell polarity protein [Source:HGNC Symbol;Acc: HGNC:30377]	8	144873090	1.45E+08	-1	q24.3
2	-283.6	8.17	28	52	21	59	100.4	DLG1	ENSP00000407531	DLG1:p , discs large MAGUK scaffold protein 1 [Source:HGNC Symbol;Acc: HGNC:2900]	3	196769431	1.97E+08	-1	q29
3	-283.6	8.17	1.8	3	1	1	95.1	DLG1	ENSP00000413238	DLG1:p , discs large MAGUK scaffold protein 1 [Source:HGNC Symbol;Acc: HGNC:2900]	3	196769431	1.97E+08	-1	q29
4	-263.7	8.17	2.9	5	1	2	103.3	DLG1	ENSP00000345731	DLG1:p , discs large MAGUK scaffold protein 1 [Source:HGNC Symbol;Acc: HGNC:2900]	3	196769431	1.97E+08	-1	q29
5	-243.2	7.83	38	50	18	37	65.5	MPP7	ENSP00000364884	MPP7:p , membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7) [Source:HGNC Symbol;Acc: HGNC:26542]	10	28339922	28623415	-1	p12.1
6	-201.9	7.95	18	25	18	47	103.2	CASK	ENSP00000354641	CASK:p , calcium/calmodulin-dependent serine protein kinase (MAGUK family) [Source: HGNC 1497]	X	41374187	41782716	-1	p11.4
7	-115.8	6.82	8.9	13	10	15	133.9	TJP2	ENSP00000366453	TJP2:p , tight junction protein 2 [Source:HGNC Symbol;Acc: HGNC:11828]	9	71736224	71870124	1	q21.11
8	-108.3	7.14	24	37	11	19	61.1	MPP6	ENSP00000222644	MPP6:p ,	7	24612887	24729159	1	p15.3

9	-81.8	6.92	18	32	7	17	46.5	LUC7L2	ENSP00000347005	LUC7L2:p , LUC7 like 2, pre-mRNA splicing factor [Source:HGNC Symbol;Acc: HGNC:21608]	7	139025105	1.39E+08	1	q34
10	-76.9	6.97	19	22	7	12	41.7	ACTB	ENSP00000349960	ACTB:p , actin beta [Source:HGNC Symbol;Acc: HGNC:132]	7	5566782	5603415	-1	p22.1
11	-56.9	7.08	27	36	6	15	21.8	LIN7C	ENSP00000278193	LIN7C:p , lin-7 homolog C (C. elegans) [Source:HGNC Symbol;Acc: HGNC:17789]	11	27516123	27528320	-1	p14.1
12	-46.6	6.55	4.3	7	1	1	38.4	LUC7L	ENSP00000337507	LUC7L:p , LUC7 like [Source:HGNC Symbol;Acc: HGNC:6723]	16	238968	279462	-1	p13.3
13	-37.5	6.81	13	17	1	1	22.9	LRRC1	ENSP00000435217	LRRC1:p ,	6	53659295	53788919	1	p12.1
14	-25.8	6.95	24	30	3	9	14.4	SUB1	ENSP00000422078	SUB1:p , SUB1 homolog, transcriptional regulator [Source:HGNC Symbol;Acc: HGNC:19985]	5	32531739	32604185	1	p13.3
15	-25.3	6.45	20	38	3	5	19.3	SRSF3	ENSP00000362820	SRSF3:p , serine and arginine rich splicing factor 3 [Source:HGNC Symbol;Acc: HGNC:10785]	6	36562145	36571209	1	p21.31
16	-21.1	6.89	36	52	3	3	10.9	C19orf33	ENSP00000301246	C19orf33:p , chromosome 19 open reading frame 33 [Source:HGNC Symbol;Acc: HGNC:16668]	19	38794801	38795649	1	q13.2
17	-20.4	6.26	3.5	5	3	3	124.9	MYO1B	ENSP00000341903	MYO1B:p ,	2	192109911	1.92E+08	1	q32.3
18	-18.4	6.72	10	22	2	4	25.5	SRSF2	ENSP00000376276	SRSF2:p , serine and arginine rich splicing factor 2 [Source:HGNC Symbol;Acc: HGNC:10783]	17	74730197	74733456	-1	q25.1
19	-16	6.61	3.9	6	2	3	57.9	SNTB2	ENSP00000338191	SNTB2:p , syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2) [Source:HGNC Symbol;Acc: HGNC ...	16	69221032	69342955	1	q22.1
20	-14.9	5.88	9.6	15	2	2	27.9	U2AF1	ENSP00000291552	U2AF1:p , U2 small nuclear RNA auxiliary factor 1 [Source:HGNC Symbol;Acc: HGNC:12453]	21	44513066	44527697	-1	q22.3
21	-14.2	6.68	18	27	2	3	16.8	CALM2	ENSP00000272298	CALM2:p ,	2	47272677	47403740	-1	p21
22	-13.2	6.13	4.3	8	2	2	59.3	RBM39	ENSP00000253363	RBM39:p , RNA binding motif protein 39 [Source:HGNC Symbol;Acc: HGNC:15923]	20	34291531	34330234	-1	q11.22
23	-12.8	6.33	16	28	2	4	23.7	ENSP00000441406	ENSP00000441406	ADP ribosylation factor like GTPase 6 interacting protein 4 [Source:HGNC Symbol;Acc: HGNC:18076]	12	123464333	1.23E+08	1	q24.31
24	-12.6	5.94	4.2	5	2	3	51.5	KRT14	ENSP00000167586	KRT14:p , keratin 14, type I [Source:HGNC Symbol;Acc: HGNC:6416]	17	39738531	39743173	-1	q21.2
25	-12.2	6.24	3.7	7	2	3	50.1	EEF1A1	ENSP00000339063	EEF1A1:p , eukaryotic translation elongation factor 1 alpha 1 [Source:HGNC Symbol;Acc: HGNC:3189]	6	74225473	74233520	-1	q13
26	-11.5	5.56	3.2	4	1	1	48.1	KRT17	ENSP00000308452	KRT17:p , keratin 17, type I [Source:HGNC Symbol;Acc: HGNC:6427]	17	39775689	39781094	-1	q21.2
27	-11.4	5.75	5.6	7	2	2	38.6	ANXA2	ENSP00000379342	ANXA2:p , annexin A2 [Source:HGNC Symbol;Acc: HGNC:537]	15	60639333	60695082	-1	q22.2
28	-11.1	5.82	4.9	7	2	2	50.1	TUBA1B	ENSP00000336799	TUBA1B:p , tubulin alpha 1b [Source:HGNC Symbol;Acc: HGNC:18809]	12	49521565	49525180	-1	q13.12
29	-10.2	5.38	0.9	1	2	2	311.5	APC	ENSP00000257430	APC:p ,	5	112043195	1.12E+08	1	q22.2
30	-10.2	5.56	5.7	9	2	2	56	RCC2	ENSP00000364585	RCC2:p ,	1	17733256	17766220	-1	p36.13
31	-7.9	5.57	1.6	2	1	1	71	HSPA6	ENSP00000310219	HSPA6:p , heat shock protein family A (Hsp70) member 6 [Source:HGNC Symbol;Acc: HGNC:5239]	1	161494036	1.61E+08	1	q23.3
32	-6.6	5.84	7.5	11	1	1	26.2	NUDT21	ENSP00000300291	NUDT21:p , nudix (nucleoside diphosphate linked moiety X)-type motif 21 [Source:HGNC Symbol;Acc: HGNC:13870]	16	56463045	56486111	-1	q12.2
33	-5	6.07	9.4	16	1	2	14.8	RPL22	ENSP00000346088	RPL22:p , ribosomal protein L22 [Source:HGNC Symbol;Acc: HGNC:10315]	1	6241329	6269449	-1	p36.31
34	-5	5.89	14	32	1	1	14.1	HIST2H2AA3	ENSP00000358158	HIST2H2AA3:p ,	1	149813505	1.5E+08	-1	q21.2
35	-4.4	4.95	0.7	1	1	1	195.3	TJP1	ENSP00000281537	TJP1:p , tight junction protein 1 [Source:HGNC Symbol;Acc: HGNC:11827]	15	29991571	30261068	-1	q13.1
36	-4.2	5.34	2.3	4	1	1	47.7	RPL4	ENSP00000311430	RPL4:p , ribosomal protein L4 [Source:HGNC Symbol;Acc: HGNC:10353]	15	66790355	66816870	-1	q22.31
37	-4.2	5.67	1.5	3	1	1	53.5	U2AF2	ENSP00000307863	U2AF2:p , U2 small nuclear RNA auxiliary factor 2 [Source:HGNC Symbol;Acc: HGNC:23156]	19	56165512	56186081	1	q13.42
38	-4.2	6.18	13	16	1	2	13.7	TAX1BP3	ENSP00000225525	TAX1BP3:p , Tax1 (human T-cell leukemia virus type I) binding protein 3 [Source:HGNC Symbol;Acc: HGNC:30684]	17	3566196	3571976	-1	p13.2

Peptide: HPV-16 PBM (16pT-2)															
rank	log(e)	log(I)	% (measured)	% (corrected)	unique	total	Mr	Identifier	Accession	Description	Chromosome	Start	End	Strand	Band
1	-80.3	7.03	18	32	7	24	46.5	LUC7L2	ENSP00000347005	LUC7L2:p , LUC7 like 2, pre-mRNA splicing factor [Source:HGNC Symbol;Acc: HGNC:21608]	7	139025105	1.4E+08	1	q34
2	-74.6	6.65	11	20	7	11	103.3	DLG1	ENSP00000345731	DLG1:p , discs large MAGUK scaffold protein 1 [Source:HGNC Symbol;Acc: HGNC:2900]	3	196769431	2E+08	-1	q29
3	-47.9	6.42	9	12	4	4	65.5	MPP7	ENSP00000364884	MPP7:p , membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7) [Source:HGNC Symbol;Acc: HGNC:26542]	10	28339922	2.9E+07	-1	p12.1
4	-35.9	6.31	7.7	9	4	6	42	ACTA1	ENSP00000355645	ACTA1:p , actin, alpha 1, skeletal muscle [Source:HGNC Symbol;Acc: HGNC:129]	1	229566992	2.3E+08	-1	q42.13
5	-29.1	6.57	17	36	3	10	25.5	SRSF2	ENSP00000376276	SRSF2:p , serine and arginine rich splicing factor 2 [Source:HGNC Symbol;Acc: HGNC:10783]	17	74730197	7.5E+07	-1	q25.1
6	-28	6.36	12	19	3	5	27.9	U2AF1	ENSP00000291552	U2AF1:p , U2 small nuclear RNA auxiliary factor 1 [Source:HGNC Symbol;Acc: HGNC:12453]	21	44513066	4.5E+07	-1	q22.3
7	-16.5	5.94	4.3	8	2	2	59.3	RBM39	ENSP00000253363	RBM39:p , RNA binding motif protein 39 [Source:HGNC Symbol;Acc: HGNC:15923]	20	34291531	3.4E+07	-1	q11.22
8	-15.9	6.32	12	23	2	4	19.3	SRSF3	ENSP00000362820	SRSF3:p , serine and arginine rich splicing factor 3 [Source:HGNC Symbol;Acc: HGNC:10785]	6	36562145	3.7E+07	1	p21.31
9	-15.5	5.84	5.6	7	2	3	38.6	ANXA2	ENSP00000379342	ANXA2:p , annexin A2 [Source:HGNC Symbol;Acc: HGNC:537]	15	60639333	6.1E+07	-1	q22.2
10	-15.5	6.09	12	22	2	5	23.7	ENSP00000441406	ENSP00000441406	ADP ribosylation factor like GTPase 6 interacting protein 4 [Source:HGNC Symbol;Acc: HGNC:18076]	12	123464333	1.2E+08	1	q24.31
11	-15.3	6.73	14	18	2	9	14.4	SUB1	ENSP00000422078	SUB1:p , SUB1 homolog, transcriptional regulator [Source:HGNC Symbol;Acc: HGNC:19985]	5	32531739	3.3E+07	1	p13.3
12	-14.6	6.15	17	29	2	3	14.8	RPL22	ENSP00000346088	RPL22:p , ribosomal protein L22 [Source:HGNC Symbol;Acc: HGNC:10315]	1	6241329	6269449	-1	p36.31
13	-13.7	5.47	13	18	2	2	18.5	CFL1	ENSP00000432660	CFL1:p ,	11	65590493	6.6E+07	-1	q13.1
14	-12.4	5.72	1.9	3	2	2	117.4	DDX46	ENSP00000416534	DDX46:p ,	5	134094469	1.3E+08	1	q31.1
15	-11.9	5.53	7.3	12	2	2	27.7	SRSF1	ENSP00000258962	SRSF1:p , serine and arginine rich splicing factor 1 [Source:HGNC Symbol;Acc: HGNC:10780]	17	56080854	5.6E+07	-1	q22
16	-11.9	5.78	11	16	2	2	26.2	NUDT21	ENSP00000300291	NUDT21:p , nudix (nucleoside diphosphate linked moiety X)-type motif 21 [Source:HGNC Symbol;Acc: HGNC:13870]	16	56463045	5.6E+07	-1	q12.2
17	-11.7	6.4	31	45	2	4	10.9	C19orf33	ENSP00000301246	C19orf33:p , chromosome 19 open reading frame 33 [Source:HGNC Symbol;Acc: HGNC:16668]	19	38794801	3.9E+07	1	q13.2
18	-10.9	5.94	3.7	7	2	2	50.1	EEF1A1	ENSP00000339063	EEF1A1:p , eukaryotic translation elongation factor 1 alpha 1 [Source:HGNC Symbol;Acc: HGNC:3189]	6	74225473	7.4E+07	-1	q13
19	-10.7	5.27	2	3	2	2	161	COL22A1	ENSP00000303153	COL22A1:p , collagen type XXII alpha 1 chain [Source:HGNC Symbol;Acc: HGNC:22989]	8	139600478	1.4E+08	-1	q24.3
20	-6.2	5.08	8	20	1	1	25.1	sp|CASB_BOVIN|	sp|CASB_BOVIN|	Beta-casein; Contains: Casoparan; Contains: Antioxidant peptide; Contains: Casohypotensin; Flags: Precursor;	nc				
21	-5.8	5.87	7.6	9	1	3	11.7	TXN	ENSP00000363641	TXN:p , thioredoxin [Source:HGNC Symbol;Acc: HGNC:12435]	9	113006091	1.1E+08	-1	q31.3
22	-5.1	5.79	2	3	1	2	49.8	TUBA3E	ENSP00000318197	TUBA3E:p , tubulin alpha 3e [Source:HGNC Symbol;Acc: HGNC:20765]	2	130949318	1.3E+08	-1	q21.1
23	-4.4	6.77	2.6	3	1	5	74.8	GIMAP8	ENSP00000305107	GIMAP8:p , GTPase, IMAP family member 8 [Source:HGNC Symbol;Acc: HGNC:21792]	7	150147718	1.5E+08	1	q36.1
24	-4.2	5.35	1.7	2	1	1	65.4	KRT2	ENSP00000310861	KRT2:p , keratin 2 [Source:HGNC Symbol;Acc: HGNC:6439]	12	53038342	5.3E+07	-1	q13.13
25	-4.1	6.03	1	1	1	1	103.2	CASK	ENSP00000354641	CASK:p , calcium/calmodulin-dependent serine protein kinase (MAGUK family) [Source: HGNC 1497]	X	41374187	4.2E+07	-1	p11.4

Peptide: HPV-16 PBM (16pT-6)															
rank	log(e)	log(l)	% (measured)	% (corrected)	unique	total	Mr	Identifier	Accession	Description	Chromosome	Start	End	Strand	Band
1	-268.2	7.5	14	19	24	44	177.6	SCRIB	ENSP00000349486	SCRIB:p , scribbled planar cell polarity protein [Source:HGNC Symbol;Acc: HGNC:30377]	8	144873090	1.4E+08	-1	q24.3
2	-218.3	7.76	21	39	20	34	103.3	DLG1	ENSP00000345731	DLG1:p , discs large MAGUK scaffold protein 1 [Source:HGNC Symbol;Acc: HGNC:2900]	3	196769431	2E+08	-1	q29
3	-212.5	7.77	1.4	3	1	4	100.4	DLG1	ENSP00000407531	DLG1:p , discs large MAGUK scaffold protein 1 [Source:HGNC Symbol;Acc: HGNC:2900]	3	196769431	2E+08	-1	q29
4	-162.8	7.31	21	28	12	19	65.5	MPP7	ENSP00000364884	MPP7:p , membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7) [Source:HGNC Symbol;Acc: HGNC:26542]	10	28339922	2.9E+07	-1	p12.1
5	-162.6	7.73	17	25	15	30	102	CASK	ENSP00000400526	CASK:p ,	X	41374187	4.2E+07	-1	p11.4
6	-102.9	7.14	22	26	9	19	41.7	ACTB	ENSP00000349960	ACTB:p , actin beta [Source:HGNC Symbol;Acc: HGNC:132]	7	5566782	5603415	-1	p22.1
7	-96.5	6.97	20	35	8	23	46.5	LUC7L2	ENSP00000347005	LUC7L2:p , LUC7 like 2, pre-mRNA splicing factor [Source:HGNC Symbol;Acc: HGNC:21608]	7	139025105	1.4E+08	1	q34
8	-66.3	6.62	12	19	7	8	61.1	MPP6	ENSP00000222644	MPP6:p ,	7	24612887	2.5E+07	1	p15.3
9	-64.4	6.96	27	36	6	12	21.8	LIN7C	ENSP00000278193	LIN7C:p , lin-7 homolog C (C. elegans) [Source:HGNC Symbol;Acc: HGNC:17789]	11	27516123	2.8E+07	-1	p14.1
10	-53.1	6.77	12	19	6	8	57.9	SNTB2	ENSP00000338191	SNTB2:p , syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2) [Source:HGNC Symbol;Acc: HGNC ...	16	69221032	6.9E+07	1	q22.1
11	-47.5	6.48	1.8	2	5	6	394.2	UTRN	ENSP00000356515	UTRN:p ,	6	144606837	1.5E+08	1	q24.2
12	-44.8	7.29	29	36	3	7	13.7	TAX1BP3	ENSP00000225525	TAX1BP3:p , Tax1 (human T-cell leukemia virus type I) binding protein 3 [Source:HGNC Symbol;Acc: HGNC:30684]	17	3566196	3571976	-1	p13.2
13	-30.2	6.77	17	36	3	6	25.5	SRSF2	ENSP00000376276	SRSF2:p , serine and arginine rich splicing factor 2 [Source:HGNC Symbol;Acc: HGNC:10783]	17	74730197	7.5E+07	-1	q25.1
14	-24	6.57	4.7	8	3	4	62.3	KRT5	ENSP00000252242	KRT5:p , keratin 5, type II [Source:HGNC Symbol;Acc: HGNC:6442]	12	52908359	5.3E+07	-1	q13.13
15	-21.5	6.42	5.6	11	3	5	50.1	EEF1A1	ENSP00000339063	EEF1A1:p , eukaryotic translation elongation factor 1 alpha 1 [Source:HGNC Symbol;Acc: HGNC:3189]	6	74225473	7.4E+07	-1	q13
16	-19.7	5.96	9.6	15	2	3	27.9	U2AF1	ENSP00000291552	U2AF1:p , U2 small nuclear RNA auxiliary factor 1 [Source:HGNC Symbol;Acc: HGNC:12453]	21	44513066	4.5E+07	-1	q22.3
17	-18.6	5.92	4.5	11	2	2	59.2	CPSF6	ENSP00000391774	CPSF6:p ,	12	69633317	7E+07	1	q15
18	-16.8	6.55	1.7	2	1	1	65.4	KRT2	ENSP00000310861	KRT2:p , keratin 2 [Source:HGNC Symbol;Acc: HGNC:6439]	12	53038342	5.3E+07	-1	q13.13
19	-16.3	5.88	4.2	5	2	3	51.5	KRT14	ENSP00000167586	KRT14:p , keratin 14, type I [Source:HGNC Symbol;Acc: HGNC:6416]	17	39738531	4E+07	-1	q21.2
20	-16.1	6.35	12	23	2	4	19.3	SRSF3	ENSP00000362820	SRSF3:p , serine and arginine rich splicing factor 3 [Source:HGNC Symbol;Acc: HGNC:10785]	6	36562145	3.7E+07	1	p21.31
21	-15.4	6.33	17	29	2	3	14.8	RPL22	ENSP00000346088	RPL22:p , ribosomal protein L22 [Source:HGNC Symbol;Acc: HGNC:10315]	1	6241329	6269449	-1	p36.31
22	-14.8	6.97	14	18	2	9	14.4	SUB1	ENSP00000422078	SUB1:p , SUB1 homolog, transcriptional regulator [Source:HGNC Symbol;Acc: HGNC:19985]	5	32531739	3.3E+07	1	p13.3
23	-14.7	5.89	5.8	8	2	2	49.8	TUBA3E	ENSP00000318197	TUBA3E:p , tubulin alpha 3e [Source:HGNC Symbol;Acc: HGNC:20765]	2	130949318	1.3E+08	-1	q21.1
24	-13	6.39	18	27	2	2	16.8	CALM2	ENSP00000272298	CALM2:p ,	2	47272677	4.7E+07	-1	p21
25	-13	6.2	2.3	3	2	3	124.9	MYO1B	ENSP00000341903	MYO1B:p ,	2	192109911	1.9E+08	1	q32.3
26	-10.8	5.75	3.5	6	2	2	72.1	DMD	ENSP00000367997	DMD:p ,	X	31137336	3.3E+07	-1	p21.1
27	-7.9	5.71	2.9	4	1	1	38.6	ANXA2	ENSP00000379342	ANXA2:p , annexin A2 [Source:HGNC Symbol;Acc: HGNC:537]	15	60639333	6.1E+07	-1	q22.2
28	-5.4	5.25	1.9	3	1	1	62	KRT9	ENSP00000246662	KRT9:p , keratin 9 [Source:HGNC Symbol;Acc: HGNC:6447]	17	39722096	4E+07	-1	q21.2
29	-5.2	5.92	7.6	9	1	2	11.7	TXN	ENSP00000363641	TXN:p , thioredoxin [Source:HGNC Symbol;Acc: HGNC:12435]	9	113006091	1.1E+08	-1	q31.3
30	-4.9	4.82	3.9	8	1	1	51.4	LUC7L3	ENSP00000425092	LUC7L3:p , LUC7 like 3 pre-mRNA splicing factor [Source:HGNC Symbol;Acc: HGNC:24309]	17	48796905	4.9E+07	1	q21.33
31	-4.9	5.77	4.5	6	1	1	22.1	PRDX1	ENSP00000361152	PRDX1:p , peroxiredoxin 1 [Source: HGNC 9352]	1	45976708	4.6E+07	-1	p34.1

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